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An integrative redescription of *Hypsibius dujardini* (Doyère, 1840), the nominal taxon for Hypsibioidea (Tardigrada: Eutardigrada)

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Abstract

A laboratory strain identified as "*Hypsibius dujardini*" is one of the best studied tardigrade strains: it is widely used as a model organism in a variety of research projects, ranging from developmental and evolutionary biology through physiology and anatomy to astrobiology. *Hypsibius dujardini*, originally described from the Île-de-France by Doyère in the first half of the 19th century, is now the nominal species for the superfamily Hypsibioidea. The species was traditionally considered cosmopolitan despite the fact that insufficient, old and sometimes contradictory descriptions and records prevented adequate delineations of similar *Hypsibius* species. As a consequence, *H. dujardini* appeared to occur globally, from Norway to Samoa. In this paper, we provide the first integrated taxonomic redescription of *H. dujardini*. In addition to classic imaging by light microscopy and a comprehensive morphometric dataset, we present scanning electron photomicrographs, and DNA sequences for three nuclear markers (18S rRNA, 28S rRNA, ITS-2) and one mitochondrial marker (COI) that are characterised by various mutation rates. The results of our study reveal that a commercially available strain that is maintained in many laboratories throughout the world, and assumed to represent *H. dujardini sensu stricto*, represents, in fact, a new species: *H. exemplaris* **sp. nov.** Redescribing the nominal taxon for Hypsibiidae, we also redefine the family and amend the definitions of the subfamily Hypsibiinae and the genus *Hypsibius*. Moreover, we transfer *H. arcticus* (Murray, 1907) and *Hypsibius conifer* Mihelčič, 1938 to the genus *Ramazzottius* since the species exhibit claws and eggs of the *Ramazzottius* type. Finally, we designate *H. fuhrmanni* as subjectively invalid because the extremely poor description precludes identifying neotype material.

Key words: Hypsibiidae, Hypsibiinae, *H. exemplaris* **sp. nov.**, *H. fuhrmanni*, model organism, polyphyly, *Ramazzottius arcticus* **comb. nov.**, *R. conifer* **comb. nov.**, Z151 strain

Introduction

In the first half of 19th century, at the start of tardigrade taxonomic classification, only eight species of water bears were recognised (Degma & Guidetti 2007; Degma *et al*. 2009–2017). The first formally described species was *Macrobiotus ursellus* (Müller, 1785), originally described as *Acarus ursellus*, and is now a *nomen nudum*. The second species was *Macrobiotus hufelandi* C.A.S Schultze (1834), redescribed classically by Bertolani & Rebecchi (1993) and later supported with DNA barcodes by Bertolani *et al.* (2011). The remaining six species were described in 1840 by Doyère and are still valid today: *Echiniscus testudo* (Doyère, 1840), *Echiniscus granulatus* (Doyère, 1840), *Echiniscus spinulosus* (Doyère, 1840), *Milnesium tardigradum* Doyère, 1840, *Hypsibius dujardini* (Doyère, 1840), and *Ramazzottius oberhaeuseri* (Doyère, 1840). Moreover, four of Doyère's species are now considered nominal species for high rank taxa, *i.e. E. testudo* (class Heterotardigrada Richters, 1926), *M. tardigradum* (order Apochela Schuster *et al.*, 1980), *H. dujardini* (superfamily Hypsibioidea Pilato, 1969), and *R. oberhaeuseri* (family Ramazzottiidae Sands *et al.*, 2008). The taxa *E. testudo*, *M. tardigradum* and *R. oberhaeuseri* have recently been redescribed using integrated taxonomy (Gąsiorek *et al.* 2017a, Michalczyk *et al.* 2012, Stec *et al.* in press, respectively). However, *H. dujardini* still awaited a modern integrated taxonomic redescription.

Many of the nominal tardigrade taxa have old descriptions (Degma *et al*. 2009–2017), which translates into significant problems for the precise diagnoses of entire species groups or genera that these species represent. This

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is particularly true for *Hypsibius dujardini*, which is the nominal species not only for the genus *Hypsibius* Ehrenberg, 1848 but for all taxonomic levels up to superfamily Hypsibioidea (Pilato, 1969) and as such needs a clear taxonomic description. Originally, Ehrenberg (1848) placed *Hypsibius oberhaeuseri* as the nominal species for the genus *Hypsibius* but Binda & Pilato (1986) re-analysed *H. oberhaeuseri* and moved it to the then new genus *Ramazzottius* Binda & Pilato, 1986. Requiring a nominal species for *Hypsibius*, in accordance with the ICZN Code, Binda & Pilato (1987) elevated *H. dujardini* to the status of a nominal species. Described from Fontainebleau near Paris, *H. dujardini* was subsequently recorded from a plethora of localities throughout the world (Kaczmarek *et al.* 2014b, 2015, 2016, McInnes 1994, McInnes *et al*. 2017). However, the results of recent integrative studies have indicated that tardigrade species may not be as widely distributed as was previously thought (*e.g.* Cesari *et al*. 2016, Gąsiorek *et al*. 2016). Any assumption that *H. dujardini* is cosmopolitan would be misleading, and without a precise redescription of *H. dujardini*, it is impossible to verify its geographic range, or the taxonomic status of records made outside the region/ecozone from which the species was originally described. A modern redescription should not only help to clarify the morphological terminology within the genus *Hypsibius* but could also be of pivotal importance in defining the hypsibioid clade comprehensively.

Moreover, a *Sciento* laboratory strain (cat. no. Z151), identified as *H. dujardini*, is today widely used as a model organism in a variety of studies, ranging from developmental biology (*e.g*. Gabriel *et al*. 2007), through anatomy (*e.g*. Hyra *et al*. 2016), physiology (*e.g*. Fernandez *et al*. 2016), cell biology (*e.g*. Hering *et al*. 2016), genetics and genomics (*e.g*. Beltrán-Pardo *et al*. 2013; Bemm *et al*. 2016), phylogenetics (*e.g*. Levin *et al*. 2016), evolutionary biology (*e.g*. Kosztyła *et al*. 2016) to astrobiology (*e.g*. Erdmann *et al*. 2017). The taxonomic status of the *Sciento* laboratory strain assigned to this species is, however, unclear because the outdated original description of *H. dujardini* lacked many key traits that would allow an unambiguous identification of the species.

In this paper, we use integrative taxonomy to redescribe *H. dujardini* from near the *locus typicus* in central France. We provide classical morphometry and images produced by both light and scanning electron microscopy. Importantly, we also present the DNA sequences of three nuclear and one mitochondrial markers. Alongside the neotype population, we also analysed the *Sciento* laboratory strain and concluded that it represents a new species. Moreover, we propose to transfer a rare Palearctic species *H. conifer* (Mihelčič, 1938) and an allegedly cosmopolitan *H. arcticus* (Murray, 1907) to the genus *Ramazzottius* (Binda & Pilato, 1986), and suppress *H. fuhrmanni* (Heinis, 1914). The transfers and the suppression allowed us to formulate a more concise diagnosis of the genus *Hypsibius*. Finally, we amend definitions of the family Hypsibiidae and subfamily Hypsibiinae.

Materials and methods

Establishing the neotype locality for *H. dujardini*. As the type locality for *H. dujardini* was small ponds in the forest of Fontainebleau near Paris (48°24'N, 2°40'E; *c*. 70 m asl), we extensively sampled several small ponds in the Château de Fontainebleau gardens. However, although we detected other typically freshwater tardigrades, such as *Dactylobiotus* Schuster, 1980 and *Isohypsibius* Thulin, 1928, we were unable to find *H. dujardini*. Therefore, we expanded our collecting area, with an additional 121 samples from the Île-de-France, in search of the nearest possible *Hypsibius* population that conformed to the original description of *H. dujardini*. We found such a population 60 km away from the *locus typicus*, in humid moss from a hollow on a tombstone in the Montmartre Cemetery, Paris (see below for more details). As species of the *H. dujardini* complex are limnoterrestrial, *i.e.* they can be found both in humid terrestrial microhabitats such as mosses and aquatic environments (the species was originally found in wet moss *Warnstorfia fluitans* (Hedw.) Loeske), we concluded this population could be designated the neotype population. In contrast to exclusively limnic eutardigrade taxa, such as *Dactylobiotus*, *Pseudobiotus* Nelson, 1980 and *Thulinius* Bertolani, 2003, species of the *H. dujardini* complex are incapable of encystation but are capable of cryptobiosis, including anhydrobiosis (*e.g*. Beltrán-Pardo *et al.* 2015, Erdmann *et al.* 2017). Since several species in the *H. dujardini* complex occur in Europe, and all superficially fit the original description of *H. dujardini*, it is not possible to state which was identified by Doyère nearly two centuries ago.

Thus, in order to comply with the article 75 of the International Code of Zoological Nomenclature (1999), we designate the neotype series and the neotype locality because: (art. 75.3.1–3) there is an urgent need to clarify the taxonomic characters (both phenotypic and genotypic) that will allow an unambiguous identification of the species (the original description is too superficial and lacks morphometric and genetic data), (art. 75.3.4) the type series

does not exist, (art. 75.3.5) the neotype series is consistent with the original description, (art. 75.3.6) the neotype series was collected as nearly as practicable from the original type locality (explained in detail above), and (art. 75.3.7) the neotype series is deposited in recognised scientific institutions.

Samples and specimens. We analysed 95 individuals of *H. dujardini* from the neotype population. All specimens were isolated from moss, collected by the third author, from a hollow in a tombstone in the Montmartre Cemetery, Paris, France (48°53'10"N, 2°19'53"E; 70 m asl) on the 23rd May 2016. The sample was processed following the protocol described by Stec *et al.* (2015). Of the 95 specimens, 65 were examined under a light microscope, so that their external and internal morphology and morphometry could be investigated. Scanning electron microscopy (SEM) was used to investigate the fine external morphology of another 16 specimens and the extracted bucco-pharyngeal apparatus of 10 specimens. The remaining four specimens were used for DNA extraction. In order to avoid misidentifications, all animals were checked under PCM before DNA extraction.

We similarly analysed 80 individuals of a clonal laboratory strain of *Hypsibius dujardini* that was originally established on 13th November 1987 by Robert McNuff from a female collected from rotting leaves in a pond in Darcy Lever, Bolton, Lancashire, England (53°33'32"N, 2°23'48"W; 75 m asl). Commercial cultures of this strain are made available by *Sciento* (under catalogue number Z151). Specimens were analysed by light microscopy (45 individuals), SEM (30 individuals), and DNA extraction (5 individuals).

For comparative analysis, an individual of *H. pallidus* (Thulin, 1911) was collected by the third author in the Słowiński National Park, Poland (54°42'53"N, 17°13'24"E; 9 m asl). The specimen corresponded well with the redescription of *H. pallidus* by Kaczmarek & Michalczyk (2009). Two specimens of *H.* cf. *convergens* (Urbanowicz, 1925) were collected by Genowefa Przybycień in an urban park at Kamienna Góra, Poland (50°46'39"N, 16°03'22"E; 490 m asl). We compared the specimens with individuals from the *terra typica* of *H. convergens* in Latvia, kindly provided by our colleauges Krzysztof Zawierucha and Łukasz Kaczmarek, and we concluded that they are morphologically and morphometrically indistinguishable. However, we think that until a redescription of *H. convergens* based on neotype material is available, all records of *H. convergens*-like animals should be considered tentative.

Additionally, we analysed two animals and three eggs of *H.* cf. *conifer* from moss on a tree growing in an old slate quarry at Hill of Foudland, Scotland (57°23'15"N, 2°39'39"W; 350 m asl). These specimens were kindly provided by Brian Blagden (Scottish Environment Protection Agency).

Microscopy and imaging. Specimens for light microscopy and morphometry were mounted on microscope slides in a small drop of Hoyer's medium according to Morek *et al.* (2016b) and examined under a Nikon Eclipse 50i phase-contrast microscope (PCM) fitted with a Nikon Digital Sight DS-L2 digital camera. Specimens for imaging in the SEM were prepared according to Stec *et al.* (2015). Bucco-pharyngeal apparatuses were extracted following the protocol of Eibye-Jacobsen (2001) as modified by Gąsiorek *et al*. (2016). Both animals and apparatuses were examined under high vacuum in a Versa 3D DualBeam SEM at the ATOMIN facility of the Jagiellonian University, Kraków, Poland. For deep structures that could not be fully focused in a single photograph, a series of 2–6 images were taken every *ca.* 0.2 µm and then assembled with Corel into a single deep-focus image.

Morphometrics. The sample size for morphometrics was chosen following the recommendations of Stec *et al.* (2016). All measurements are given in micrometres (µm). Structures were measured only if their orientations were suitable. Body length was measured from the anterior end of the body to the posterior end, excluding the hind legs. Terminology for the structures within the bucco-pharyngeal apparatus and for the claws follows that of Pilato & Binda (2010) and Gąsiorek *et al*. (2017b). Macroplacoid length sequence is given according to Kaczmarek *et al.* (2014a). Claws were measured following Beasley *et al.* (2008). The *pt* ratio is the ratio of the length of a given structure to the length of the buccal tube, expressed as a percentage (Pilato 1981) and is presented here in italics. Morphometric data were handled using version 1.2 of the "Parachela" template, which is available from the Tardigrada Register (Michalczyk & Kaczmarek 2013).

Genotyping. DNA was extracted from individual animals using Chelex 100 resin (Casquet *et al.* 2012; Stec *et al.* 2015). We sequenced four DNA fragments that differed in their effective mutation rates: a small ribosome subunit (18S rRNA), a large ribosome subunit (28S rRNA), an internal transcribed spacer (ITS-2) and cytochrome oxidase subunit I (COI). Both **18S rRNA** and **28S rRNA** are nuclear markers that can be applied in phylogenetic analyses to investigate high taxonomic levels (*e.g.* Field *et al.* 1988). **ITS-2** is a non-coding

nuclear fragment with high evolution rates that is suitable for both intra-specific comparisons and comparisons between closely related species (*e.g.* Gąsiorek *et al.* 2016). **COI** is a protein-coding mitochondrial marker that is widely used as a standard barcode gene of intermediate effective mutation rate (*e.g.* Bertolani *et al.* 2011). All fragments were amplified and sequenced according to the protocols described by Stec *et al.* (2015); primers and original references for specific PCR programmes are listed in Table 1. As universal metazoan primers for COI, *i.e.* LCO1490, HCO2198 (Folmer *et al*. 1994), and HCOoutout (Prendini *et al*. 2005), did not amplify the fragments, we designed new primers based on three parachelan mitochondrial genomes (COI_Para_F, homologous with LCO1490) and fifteen eutardigrade COI sequences (COI_Eutar_Rr, homologous with HCO2198); see Table 1. Sequencing products were read with the ABI 3130xl sequencer at the Molecular Ecology Laboratory of the Institute of Environmental Sciences at the Jagiellonian University. Sequences were processed using version 7.2.5 of BioEdit (Hall 1999).

TABLE 1. Primers and references for specific protocols for amplification of the four DNA fragments sequenced in the study.

DNA fragment	Primer name	Primer direction	Primer sequence (5'-3')	Primer source	PCR programme
18S rRNA	SSU01_F	forward	AACCTGGTTGATCCTGCCAGT	Sands *et al.* (2008)	Zeller (2010)
	SSU82_R	reverse	TGATCCTTCTGCAGGTTCACCTAC		
28S rRNA	28SF0001	forward	ACCCVCYNAATTTAAGCATAT	Mironov *et al.* (2012)	Mironov *et al.* (2012)
	28SR0990	reverse	CCTTGGTCCGTGTTTCAAGAC		
ITS-2	ITS2_Eutar_Ff	forward	CGTAACGTGAATTGCAGGAC	Stec *et al.* (in press)	Stec *et al.* (in press)
	ITS2_Eutar_Rr	reverse	TGATATGCTTAAGTTCAGCGG		
COI	COI_Para_F	forward	GGTCAACAAATCATAAAGATATTGG	**present study**	Michalczyk *et al.* (2012)
	COI_Eutar_Rr	reverse	TAAACTTCTGGGTGACCRAARAAYCA		

18S rRNA sequences of all available hypsibiid taxa were used to reconstruct the phylogeny (Table 2). Moreover, used all four molecular markers for the genetic comparison of *H. dujardini* with other *Hypsibius* species and the closely related genus *Borealibius* (Pilato *et al*., 2006). Our ITS-2 sequences appear to represent the first reported for the genus *Hypsibius*. We used all published sequences for *Hypsibius* that were available from GenBank and of high quality (*i.e*. without numerous unknown nucleotides) and were associated with published taxonomic identifications (Table 3). Sequences were aligned with MAFFT version 7 (Katoh *et al*. 2002, in press, Katoh & Toh 2008, Kuraku *et al*. 2013). The aligned sequences were then trimmed to 758, 740, and 579 bp (for the 18S rRNA, 28S rRNA, and COI fragment, respectively). MEGA7 (Kumar *et al*. 2016) was then used to calculate uncorrected pairwise distances and, for COI, to translate nucleotide sequences to polypeptides and test for eventual pseudogenes.

Phylogenetic analyses. Sequences were aligned using default settings of MAFFT. The obtained 18S rRNA and COI alignments were edited and checked manually in BioEdit and then trimmed to 768 and 579 bp, respectively. As the COI is a protein coding gene, before partitioning, we divided our alignment into three data blocks constituting three separated codon positions. Based on PartitionFinder version 2.1.1 (Lanfear *et al.* 2016), under the Bayesian Information Criterion (BIC), the best scheme of partitioning and substitution models were chosen for posterior phylogenetic analysis. First we ran the analysis to test all possible models implemented in the program. As the best-fit partitioning scheme, PartitionFinder suggested retaining the three predefined partitions separately. The best-fit models for these partitions were: SYM+G for the first codon position, TVM+I+G for the second codon positions and TRN+G for the third codon position.

Bayesian inference (BI) marginal posterior probabilities were calculated for 18S rRNA using MrBayes v3.2 (Ronquist & Huelsenbeck 2003). Random starting trees were used and the analysis was run for ten million generations, sampling the Markov chain every 1000 generations. An average standard deviation of split frequencies of <0.01 was used as a guide to ensure the two independent analyses had converged. The program Tracer v1.3 (Rambaut *et al.* 2014) was then used to ensure Markov chains had reached stationarity and to determine the correct 'burn-in' for the analysis, which was the first 10% of generations. The ESS values were >>200. A consensus tree was obtained after summarising the resulting topologies and discarding the 'burn-in'.

For the BI consensus tree, clades recovered with posterior probability (PP) between 0.95 and 1 were considered well supported, those with PP between 0.90 and 0.94 were considered moderately supported, and those with lower PP were considered unsupported. Additionally, for both markers, we also ran a maximum likelihood (ML) analysis with PhyML v. 3.0 (Guindon *et al*. 2010) with the automatic model selection by SMS (Lefort *et al*. 2017). The branch supports were calculated using approximate likelihood ratio test (aLRT) (Anisimova & Gascuel 2006). ML supports below 0.7 were considered insignificant. The final consensus trees were viewed and visualised by FigTree v.1.4.3 available from http://tree.bio.ed.ac.uk/software/figtree.

Data deposition. Raw data underlying the redescription of the neotype *H. dujardini sensu stricto* and the description of *H. exemplaris* **sp. nov.** are deposited in the *Tardigrada Register* (Michalczyk & Kaczmarek 2013) under www.tardigrada.net/register/0048.htm and www.tardigrada.net/register/0049.htm, respectively. DNA sequences were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank). Additionally, raw morphometric data are provided as Supplementary Materials 1 and 2 and p-distances as Supplementary Materials 3.

TABLE 2. List of GenBank accession numbers for Hypsibiidae 18S rRNA sequences used for constructing the phylogenetic tree of the family in the present study (new sequences are marked in bold).

Species	18S rRNA	Reference
Acutuncus antarcticus	AB753858	Kagoshima *et al*. (2013)
Adropion belgicae	HQ604925	Bertolani *et al*. (2014)
Adropion scoticum	HQ604922, **MG833237**	Bertolani *et al*. (2014), **present study**
Astatumen trinacriae	FJ435733, HQ604922	Guil & Giribet (2012), Bertolani *et al*. (2014)
Borealibius zetlandicus	HQ604924	Rebecchi *et al*. (2009), Bertolani *et al*. (2014)
Diphascon higginsi	HQ604932	Bertolani *et al*. (2014)
Diphascon pingue	FJ435736, HQ604937	Guil & Giribet (2012), Bertolani *et al*. (2014)
Diphascon puniceum	EU266949	Sands *et al*. (2008)
Hypsibius convergens	FJ435726	Guil & Giribet (2012)
***Hypsibius dujardini* s.s.**	**MG777532**	**present study**
***Hypsibius exemplaris* sp. nov.**	**MG800327**	**present study**
Hypsibius klebelsbergi	KT901827	Dabert *et al*. (2014, 2015)
Hypsibius pallidus	HQ604945	Bertolani *et al*. (2014)
Hypsibius scabropygus	AM500649	Dabert *et al*. (2014)
Mesocrista revelata	KU528627	Gąsiorek *et al*. (2016)
Mesocrista spitzbergensis	KX347532	Gąsiorek *et al*. (2016)
Pilatobius nodulosus	HQ604934	Bertolani *et al*. (2014)
Pilatobius patanei	HQ604935	Bertolani *et al*. (2014)
Pilatobius ramazzottii	HQ604939	Bertolani *et al*. (2014)
Pilatobius recamieri	KX347526	Gąsiorek *et al*. (2017)
Platicrista angustata	HQ604948	Bertolani *et al*. (2014)
outgroup (Macrobiotidae):		
Macrobiotus macrocalix	HQ604976	Bertolani *et al*. (2014)
Macrobiotus paulinae	KT935502	Stec *et al*. (2015)

TABLE 3. List of GenBank accession numbers for Hypsibiinae and sequences used for molecular analyses (p-distances and COI-based phylogeny of the subfamily) in the present study (new sequences are marked in bold).

Species	18S rRNA	28S rRNA	ITS-2	COI	Reference
H. convergens	FJ435726	FJ435771	–	FJ435798	Guil & Giribet (2012)
***H. dujardini* s.s.**	**MG777532**	**MG777533**	**MG777531**	**MG818723**	**present study**
***H. exemplaris* sp. nov.**	**MG800327**	**MG800337**	**MG800336**	**MG818724**	**present study**
H. klebelsbergi	KT901827	KC582835	–	KT901831, 4	Dabert *et al*. (2014, 2015)
H. pallidus	HQ604945	–	–	–	Bertolani *et al*. (2014)
H. scabropygus	AM500649	–	–	–	Dabert *et al*. (2014)
B. zetlandicus	HQ604924	–	–	FJ184601	Rebecchi *et al*. (2009), Bertolani *et al*. (2014)
outgroup:					
M. spitzbergensis	–	–	–	KX347535	Gąsiorek *et al*. (2016)

Results

Taxonomic accounts

Phylum: Tardigrada Doyère, 1840

Class: Eutardigrada Richters, 1926

Order: Parachela Schuster, Nelson, Grigarick and Christenberry, 1980

Superfamily: Hypsibioidea Pilato, 1969 (in Marley *et al.* 2011)

Amended diagnosis. Eutardigrades with asymmetrical claws (2-1-2-1) and pseudolunulae at claw bases or without any cuticular structures under the basal parts. Hooked or broad-ridged apophyses for the insertion of the stylet muscles. Herbivorous or microbivorous (Guidetti *et al*. 2012).

Composition. Calohypsibiidae Pilato, 1969, Hypsibiidae Pilato, 1969, Microhypsibiidae Pilato, 1998, Ramazzottiidae Sands *et al*., 2008.

Family: Hypsibiidae Pilato, 1969

Amended diagnosis. Eutardigrades without cephalic papillae (*sensu* structures present *e.g*. in *Halobiotus* Kristensen, 1982; see Møbjerg *et al*. 2007) and elliptical organs. Claws of the *Hypsibius* type, *i.e.* asymmetrical both with respect to the sequence of primary and secondary branches (2-1-2-1) and with respect to the size, with external and posterior claws being always clearly larger than internal and anterior claws. Accessory points symmetrical. Two types of bucco-pharyngeal apparatuses: with the buccal tube rigid over its entire length (Hypsibiinae) or with a rigid anterior buccal tube followed by a flexible posterior pharyngeal tube (all remaining subfamilies).

Composition. Diphasconinae Dastych, 1992, Hypsibiinae Pilato, 1969, Itaquasconinae Rudescu, 1964, Pilatobiinae Bertolani *et al*., 2014

Subfamily: Hypsibiinae Pilato, 1969

Diagnosis. Hypsibiids without pharyngeal tube. Smooth eggs laid in exuviae.

Composition. *Hypsibius* Ehrenberg, 1848, *Borealibius* Pilato *et al*., 2006

Genus: *Hypsibius* Ehrenberg, 1848

Amended diagnosis. Six weakly outlined peribuccal lobes present. Apophyses for the insertion of stylet muscles in the shape of symmetrical hooks; and with well-developed caudal processes pointing diagonally (backwards and sideways, see Figs 19–20). Pharyngeal apophyses and placoids present. Stylet furcae with the triangular base, thin arms and enlarged apices (*sensu* Pilato & Binda 2010). Claws of the *Hypsibius* type. Smooth eggs laid in exuviae (see Remarks below).

Remarks. Only three (7%) species that are currently attributed to the genus lay ornamented eggs: *H. fuhrmanni*, *H. arcticus*, and *H. conifer*. However, the original descriptions of the first species is now considered extremely limited, and the latter two species represent, in our opinion, a different genus. Thus, we designate *H. fuhrmanni* as subjectively invalid and we transfer *H. arcticus*, and *H. conifer* to the genus *Ramazzottius*:

Hypsibius fuhrmanni, originally described from Colombia as *Macrobiotus fuhrmanni* (Heinis 1914), exhibits a mixture of taxonomic traits. The claws were described by Heinis (1914) as of the *Diphascon*-type, but the drawing provided (fig. 38 in Heinis 1914) is very schematic and does not allow a confident identification of the claw type. The eggs appear to be similar to those laid by some of the *Ramazzottius* or *Hebesuncus* spp., and the bucco-pharyngeal apparatus of an unknown affinity but definitely not of the *Hypsibius* type. This unlikely combination suggests that the description may have been based on two different genera, neither of which represents *Hypsibius* (according to the current diagnosis of the genus). Therefore, due to this this evident confusion, lack of type material, or chance of identifying neotype material, we designate *H. fuhrmanni* as subjectively invalid.

Hypsibius arcticus was originally described from Svalbard and Franz Joseph Land as *Macrobiotus arcticus* (Murray, 1907a), and later transferred by Thulin (1911) to the genus *Hypsibius*. The species has a long history of confusion (described in detail in Dastych 1991) that started with Murray himself, who was not sure whether some of his records represented *H. arcticus* or other species (*e.g.* Murray 1907b, 1911). Nevertheless, he reported the species from numerous localities throughout the world, even though very often he had collected only eggs or only animals and he frequently doubted his own identifications.: In addition to the type locality, *H. arcticus* was allegedly found in Africa (Murray 1907c, 1913a), Europe (Murray 1907b, 1911), Antarctica, North and South America, Australia and New Zealand (Murray 1910, Murray 1913b). Other authors either repeated Murray's records and illustrations (*e.g.* Marcus 1936, Cuénot 1932, Ramazzotti & Maucci 1983) or increased the confusion by adding uncertain records (*e.g.* Richters 1911 who probably reported a misidentified *Murrayon hastatus* (Murray, 1907b); see Dastych 1991). Murray (1910), realising that the original description was not very detailed, attempted to redescribe the species using Antarctic samples. Given that the *locus typicus* of *H. arcticus* is on the other side of the globe, Murray (1910) cannot be considered a valid redescription of the species according to modern taxonomic standards. Moreover, numerous records of *H. arcticus* from the Antarctic (reviewed in Dastych 1991) are now considered as invalid or representing *Acutuncus antarcticus* (Richters, 1904). Such a wide distribution reported in older literature combined with only occasional and dubious records in the recent literature (see Kaczmarek *et al.* 2015, 2016 and McInnes *et al.* 2017) suggests that a variety of taxa have most likely been incorrectly as attributed to *H. arcticus*. Thus, we suggest that the Franz Joseph Land record (Murray 1907a) should be considered as *locus typicus* and the only certain record for *H. arcticus*.

The original description of *H. arcticus* (Murray 1907a) was based on two eggs of different size. The smaller egg from Franz Joseph Land contained a mature embryo and was used by Murray (1907a) to draw the details of the buccal apparatus and claws (figs 5d–e in Murray 1907a). However, the larger egg, from Svalbard, might represent an egg of *Murrayon hastatus* (Murray, 1907b). Drawings in the original description of *H. arcticus* (figs 5d–e in Murray 1907a) strongly suggest some of the key characteristics of the genus *Ramazzottius*. Specifically, an ornamented and freely laid egg, external and posterior claws with extremely elongated primary branches, buccal apparatus with two granular macroplacoids and no microplacoids or septulum (see figs 5d–e in Murray 1907a). In our opinion, these traits place *H. arcticus* in the genus *Ramazzottius* rather than in *Hypsibius*. Moreover, Murray (1907b) originally described the Scottish record of *H. arcticus* as "*Macrobiotus sp.*? near *M. oberhäuseri"* and figs 27a–d in Murray (1907b) leave no doubt that the depicted tardigrade is a ramazzottiid (especially the claws depicted in fig. 27c, with an evident flexible connector at the base of the posterior primary branch, are very characteristic for the family Ramazzottiidae). Although, as stated above, we do not consider the Scottish record as valid, the fact that Murray after reanalysing the record classified it as *H. arcticus*, suggests that the embryo on which the original description was based, also exhibited a similar (*i.e.* ramazzottiid) morphology.

It is important to remember that at the time when Thulin (1911) transferred *M. arcticus* to *Hypsibius*, the genus was very broadly defined and comprised numerous genera (including *Ramazzottius*) that have since been classified into several parachelan families. In other words, the designation of *M. arcticus* as *H. arcticus* is a historical artefact, and this may not be the only example of a ramazzottiid still bearing a now historically incorrect classification within *Hypsibius* (possible candidates include: *H. calcaratus* Bartoš, 1935, *H. hypostomus* Barto, 1935 and *H. macrocalcaratus* Beasley, 1988).

We, therefore, designate the species as *Ramazzottius arcticus* **comb. nov.**, pending a modern redescription based on neotype material from the Arctic.

The original description of ***Hypsibius conifer*** (Mihelčič 1938) clearly indicated *Ramazzottius* type claws and eggs. Moreover, we found several individuals and eggs that, based on the original description, we identified as *H.* cf. *conifer*, and our observations confirm that the species is more similar to *Ramazzottius* than to *Hypsibius* (Figs 32–36). The species was described decades prior to the erection of the genus *Ramazzottius*, thus we consider its current taxonomic position as a historical artefact. Therefore, we designate this species as *Ramazzottius conifer* **comb. nov.**, pending a modern redescription based on neotype material (for more details on the morphology of the species see below and Figs 32–36).

Thus, with the exclusion of the three abovementioned species from the *Hypsibius* genus, all currently reported *Hypsibius* spp. lay smooth eggs into shed exuviae.

Etymology. Ehrenberg (1848) did not justify the etymology for the genus *Hypsibius*. We conjecture that he intended to distinguish *Hypsibius* from *Macrobiotus* Schultze, 1834, on the basis of claw morphology, and used the Greek word "hypso" (ὕψος; literally: height, high) to emphasise the elongated primary branches that are typical of *Hypsibius* type claws; differentiating them from much more symmetrical *Macrobiotus* claws.

Composition. 42 species (including *H. exemplaris* **sp. nov.** described below, and excluding the three species discussed above), with *H. dujardini* being the type species (Binda & Pilato 1987).

***Hypsibius dujardini* (Doyère, 1840)**

Unidentified species: Forêt de Fontainebleau; Dujardin (1838)
Macrobiotus dujardin; ***locus typicus***: Forêt de Fontainebleau (*ca*. 48°24'N, 2°42'E); Doyère (1840)
M. lacustris, *M. palustris*; Paris and Fontainebleau; Dujardin (1851)
M. tetradactylus; Paris; Lance (1896)
Hypsibius dujardini; Fontainebleau; Cuénot (1932)

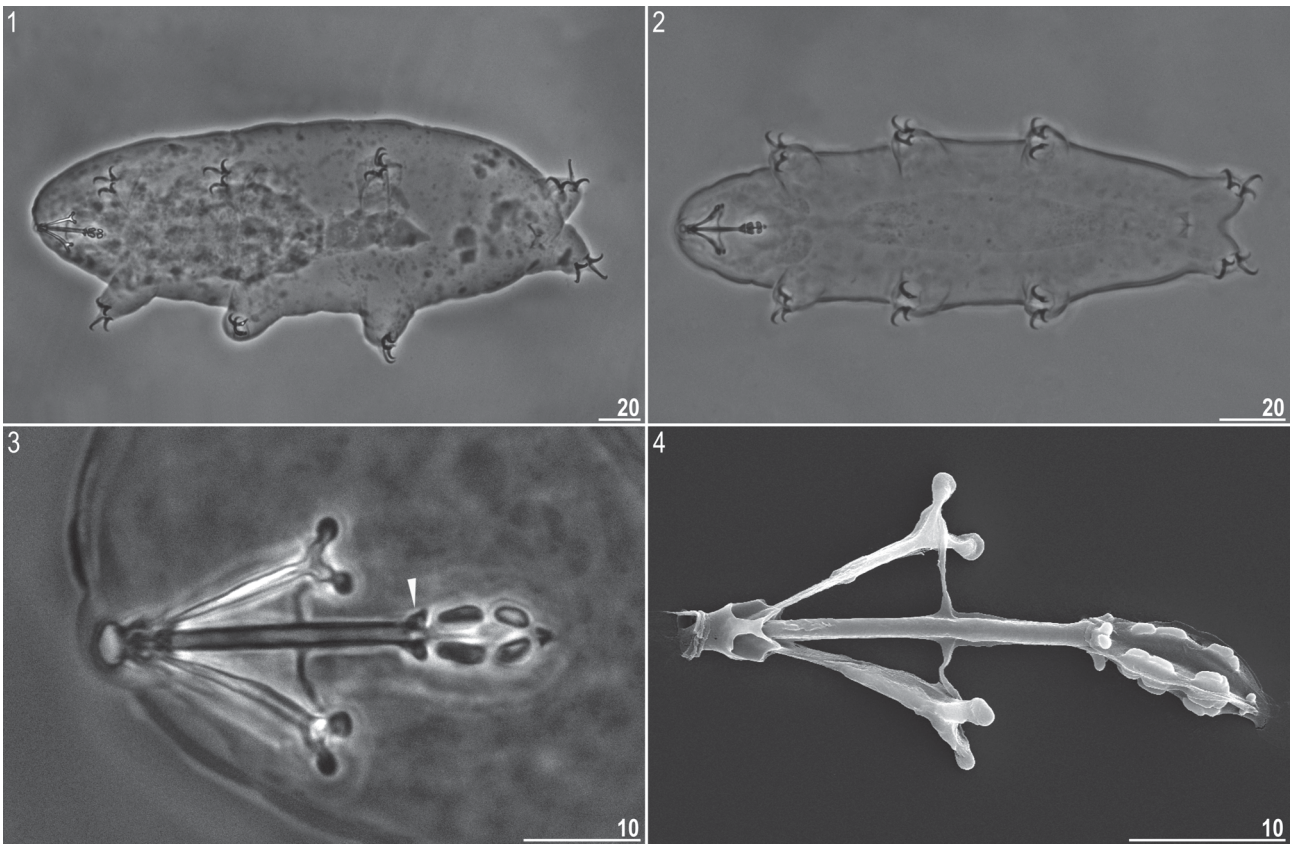
Neotype locality. 48°53'10''N, 2°19'53''E; 70 m asl: France, Île-de-France, Paris, Montmartre Cemetery; humid moss from a wet hollow in a shaded tombstone.

Material examined. Neotype and 80 neoparatypes from Paris (neotype and 64 neoparatypes on slides FR.055.01–17 and 16 neoparatypes on an SEM stub) deposited in the Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków, Poland. Neoparatypes, mounted in Hoyer's medium, include 4 juveniles, 6 simplex specimens and 3 moulting specimens with exuviae.

Integrative redescription. *Animals (see Table 4 for measurements):* Body stubby, whitish, covered with smooth cuticle, both under PCM and SEM. Eyes present in live animals, but prone to dissolution in Hoyer's medium (Figs 1–2). Buccal apparatus of the *Hypsibius* type (Figs 3–4). Mouth opening surrounded by a thin peribuccal ring without papulae or papillae. The oral cavity armature visible only under SEM, consists of 3–4 rows of minute conical teeth located on the ring fold (Fig. 17). Two distinct porous areas on the lateral sides of the crown are visible in SEM only. Stylet furcae of the *Hypsibius* type (Figs 3–4, 22). Roundish muscle pharynx with eminent pharyngeal apophyses (in juveniles almost as long as macroplacoids; Fig. 2), two oval macroplacoids and the septulum (Figs 3, 23). Macroplacoid length sequence 2<1. In PCM, the first macroplacoid with a subtle central constriction (not always visible), second macroplacoid smooth (Fig. 3). Under SEM, both macroplacoids with clear constrictions: the first macroplacoid constricted anteriorly, the second—subterminally (Fig. 23, arrowheads). Claws of the *Hypsibius* type, with obvious accessory points on the primary branches (Figs 5–8). A clear septum dividing the claw into the basal and the branch portion; septum between the primary and the secondary branch typically less visible (Figs 5–6). In juveniles, claws have a uniform structure, without septa (Fig. 2). Internal and anterior basal claws with broad, robust trunks (Figs 6–8), anterior claws with pseudolunulae (Figs 6, 8, empty arrowheads). Between the posterior and the anterior claw a short longitudinal bar is present. The bar is evidently closer to the posterior claw, but it is always separated from the claw base (Fig. 6, arrowhead). Cuticular bars on legs I–III absent.

TABLE 4. Measurements [in µm] of selected morphological structures of individuals of neotype *Hypsibius dujardini s.s.* (Doyère, 1840) mounted in Hoyer's medium. N—number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation.

CHARACTER	N	RANGE		MEAN		SD		Neotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	30	134 – 339	*843 – 1372*	289	*1175*	45	*110*	289	*1204*
Buccal tube									
Buccal tube length	30	15.9 – 27.5	–	24.5	–	2.4	–	24.0	–
Stylet support insertion point	30	9.1 – 17.4	*57.2 – 64.2*	15.1	*61.5*	1.7	*1.5*	15.0	*62.5*
Buccal tube external width	30	1.1 – 2.5	*6.9 – 10.2*	2.1	*8.6*	0.3	*0.8*	2.3	*9.6*
Buccal tube internal width	30	0.3 – 1.4	*1.9 – 5.7*	1.0	*3.9*	0.2	*0.7*	0.9	*3.8*
Placoid lengths									
Macroplacoid 1	30	2.1 – 5.1	*13.2 – 19.9*	4.1	*16.6*	0.6	*1.6*	3.9	*16.3*
Macroplacoid 2	30	1.7 – 3.9	*9.3 – 15.2*	3.1	*12.7*	0.5	*1.3*	3.0	*12.5*
Septulum	30	0.7 – 1.7	*3.3 – 6.5*	1.3	*5.1*	0.3	*0.8*	1.0	*4.2*
Macroplacoid row	30	4.6 – 9.6	*26.4 – 37.4*	8.2	*33.2*	1.1	*2.5*	8.0	*33.3*
Claw 1 lengths									
External base	26	2.0 – 4.8	*12.6 – 20.0*	4.0	*16.2*	0.6	*2.0*	4.0	*16.7*
External primary branch	17	8.3 – 11.7	*34.9 – 47.4*	9.7	*38.7*	0.9	*3.5*	10.0	*41.7*
External secondary branch	23	6.3 – 7.9	*25.6 – 32.3*	7.1	*28.3*	0.6	*1.9*	7.4	*30.8*
Internal base	28	1.2 – 4.5	*7.5 – 18.2*	3.6	*14.3*	0.6	*2.0*	3.6	*15.0*
Internal primary branch	14	5.2 – 7.7	*24.0 – 30.8*	6.8	*27.1*	0.7	*2.3*	7.3	*30.4*
Internal secondary branch	14	3.0 – 5.9	*16.5 – 22.9*	5.0	*20.0*	0.7	*1.6*	4.9	*20.4*
Claw 2 lengths									
External base	26	2.5 – 5.3	*13.7 – 22.6*	4.4	*17.8*	0.6	*2.2*	4.5	*18.8*
External primary branch	21	6.0 – 11.5	*33.0 – 46.0*	9.6	*39.7*	1.5	*3.5*	10.7	*44.6*
External secondary branch	25	4.0 – 8.6	*22.0 – 32.8*	7.1	*28.8*	1.0	*2.3*	7.1	*29.6*
Internal base	28	2.0 – 4.6	*12.4 – 18.0*	3.8	*15.4*	0.6	*1.6*	4.1	*17.1*
Internal primary branch	16	6.7 – 9.4	*27.2 – 36.1*	8.0	*31.3*	0.8	*2.4*	7.8	*32.5*
Internal secondary branch	20	3.2 – 6.6	*17.6 – 24.3*	5.4	*21.5*	0.7	*2.2*	5.6	*23.3*
Claw 3 lengths									
External base	28	2.7 – 6.2	*15.3 – 22.7*	4.6	*18.9*	0.7	*2.0*	4.8	*20.0*
External primary branch	21	5.9 – 11.5	*32.4 – 45.6*	9.7	*39.8*	1.5	*3.3*	?	*?*
External secondary branch	27	4.1 – 8.3	*25.3 – 32.9*	7.1	*28.8*	0.8	*1.9*	6.8	*28.3*
Internal base	25	2.3 – 4.6	*13.2 – 18.6*	3.8	*15.6*	0.5	*1.6*	3.7	*15.4*
Internal primary branch	13	4.6 – 9.0	*28.0 – 35.4*	7.8	*31.6*	1.1	*2.5*	8.5	*35.4*
Internal secondary branch	16	3.4 – 6.9	*19.3 – 26.8*	5.6	*22.8*	0.9	*2.0*	5.2	*21.7*
Claw 4 lengths									
Anterior base	30	2.6 – 5.0	*13.4 – 20.2*	4.2	*16.9*	0.6	*1.8*	4.8	*20.0*
Anterior primary branch	25	5.0 – 9.1	*27.6 – 35.2*	7.7	*31.8*	1.0	*2.1*	7.9	*32.9*
Anterior secondary branch	13	3.7 – 6.5	*20.3 – 26.7*	5.5	*22.7*	0.9	*1.6*	6.4	*26.7*
Posterior base	28	3.3 – 6.3	*15.6 – 26.0*	5.0	*20.3*	0.7	*2.3*	4.9	*20.4*
Posterior primary branch	25	6.5 – 14.0	*40.9 – 56.3*	12.2	*49.4*	1.6	*4.0*	13.5	*56.3*
Posterior secondary branch	24	4.5 – 8.7	*26.4 – 34.0*	7.4	*30.5*	1.0	*2.2*	7.3	*30.4*



FIGURES 1–4. *Hypsibius dujardini* (Doyère, 1840): **1**—adult habitus (ventrolateral view, PCM, neotype); **2**—*ex ovo* juvenile habitus (ventral view, PCM, neoparatype); **3**—bucco-pharyngeal apparatus (dorso-ventral projection, the arrowhead indicates large pharyngeal apophyses, PCM, neoparatype); **4**—bucco-pharyngeal apparatus (ventral view, SEM, neoparatype). All scale bars in µm.

Eggs: Roundish and smooth, deposited in exuviae (up to twelve per clutch were isolated from the moss sample).

Molecular markers: The sequences for all four DNA markers and four specimens (isogenophores) were of a very good quality. All markers were represented by a single haplotype:

The **18S rRNA** sequence (MG777532), 1,729 bp long:

AGATTAGCCATGCATGTCTCAGTACTTGCTTTAACAAGGCGAAACCGCGAATGGCTCATTAAATCAGTTATGGTTCACTA
GATCGTACAGTTTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCAACCAGTCCGTGCCCTCGTGGTGCGGACG
CAGTTATTTGCCCAAGACCAATCCGGCCCTCGGGTCGTTCAATTGGTGACTCTGAATAACCGAAGCAGAGCGCTTAGTCT
CGTACTGGCGCCAGATCTTTCAAGTGTCTGACTTATCAGCTTGTTGTTAGGTTATGTTCCTAACAAGGCTCTCACGGGTA
ACGGAGTGTCAGGGCCCGACACCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCGCAAAT
TACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATGCGAGAGCTTTTAGCTTCTCGTAATCGGAATGGGTAC
ACTTTAAATCCTTTAACGAGGATCTATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTA
TATTAAAGTTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGGGTAGTCGATGGACGGTGCTTCGTAAGGAGCTACTGCCC
GTTCGGCACCACAGCCCGGCCATGTCTTGCATGCTCTTCACTGAGTGTGCTTGGCGACCGGAACGTTTACTTTGAAAAAA
TTAGAGTGCTCAAAGCAGGCGTTAAGCCTTGTATAATGGTGCATGGGATAATGGAATAAGATTTTTGGCTTGTTCTGTTG
GTTTTAGAGTCAGAAGTAATGATAAATAGGAACAGACGGGGGGCATTCGTATTGCGGCGTTAGAGGTGAAATTCTTGGAT
CGTCGCAAGAACGCACTACTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGAGGTTCGAAGG
CGATCAGATACCGCCCTAGTTCTAACCATAAACGATGCCAACCAGCGATCCGTCGGTGTTTATTTGATGACTCGACGGGC
AGCTTCCGGGAAACCAAAGTGCTTAGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAATTGACGGAAGGG
CACCACCAGGAGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGAAAACTTACCCGGCCCGGACACTGTAAGGATTGAC
AGATTGAGAGCTCTTTCTTGATTCGGTGGGTGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGCGATTTGTCTGGTTAATT
CCGATAACGAACGAGACTCTAGCCTGCTAAATAGCCAACTGATCCGCAGCGTCGGTTGCTTATAATGCTTCTTAGAGGGA
CAGGCGGCTTCCAGTCGCACGAGATTGAGCAATAACAGGTCTGTGATGCCCTTAGATGTCCGGGGCCGCACGCGCGCTAC
ACTGAAGGAATCAACGTGCTTTCTTACCTTGGCCGGAAGGCCTGGGGAATCCGATGAAACTCCTTCGTGATTGGGATTGA
GCTTTGTAACTATCGCTCATGAACGAGGAATTCCCAGTAAGCGCGAGTCATAAGCTCGCGTTGATTACGTCCCTGCCCTT
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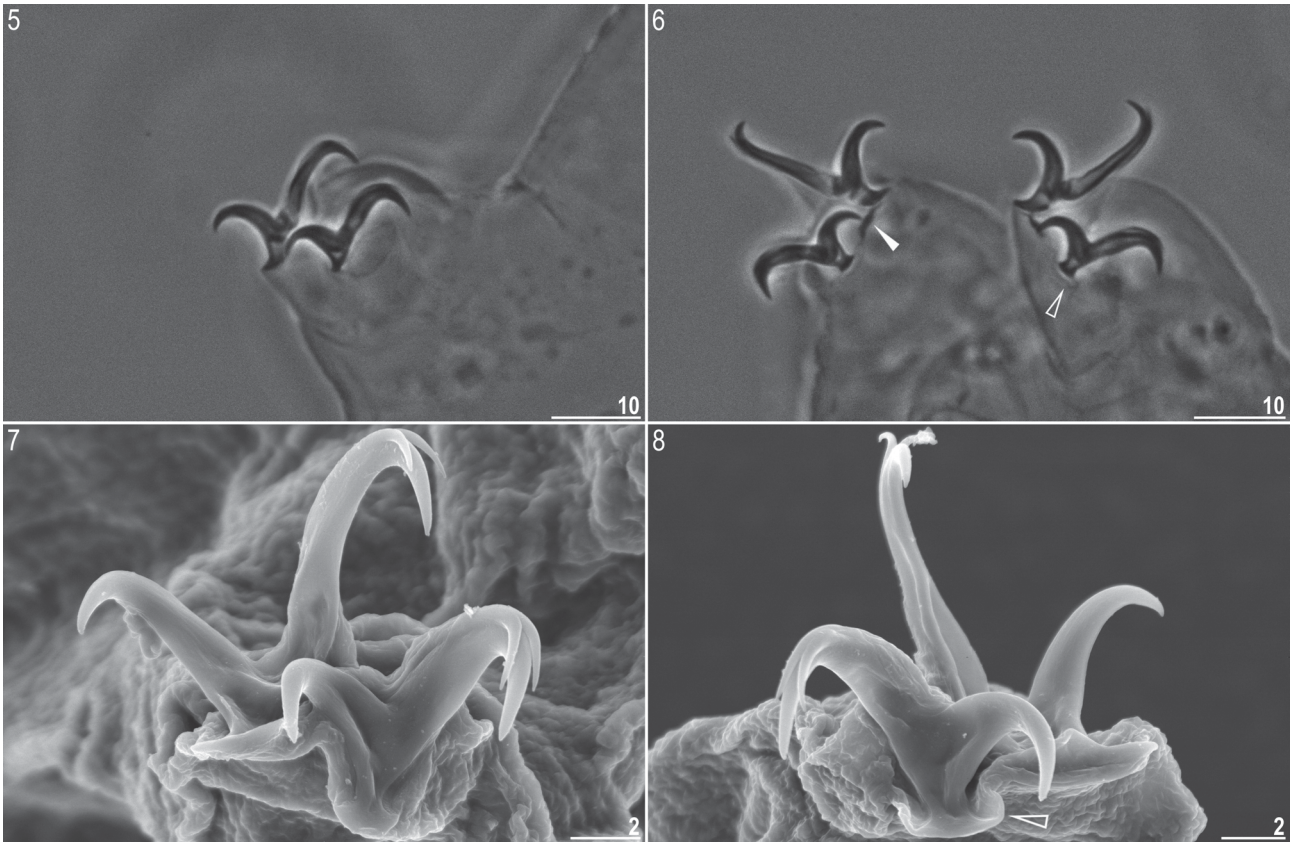
GCCTCGTTTGGTTGGAAAGAAGACCAAACTGATCATTAGAGGAAGTAAA



FIGURES 5–8. *Hypsibius dujardini* (Doyère, 1840), claws: **5**—claws I (PCM, neoparatype); **6**—claws IV, the arrowhead indicates the longitudinal bar at the posterior claw base, and the empty arrowhead indicates the pseudolunula at the anterior claw base (PCM, neoparatype); **7**—claws II (SEM, neoparatype); **8**—claws IV, the empty arrowhead indicates the pseudolunula at the anterior claw base (SEM, neoparatype). All scale bars in μm.

The **28S rRNA** sequence (MG777533), 786 bp long:

AATTTAAGCATATTACTAAGCGGAGGAAAAGAAACCAACGGGGATTCCCATAGTAACTGCGAGTGAAAGGGGAAAAGCCC
AGCGCCGAATCCTGCCGCTGGAGACGGTGGCAGGAACTGTGGCGTGAAGATGGTGTCTATCGGTGTGGCTCGCTCGCGTA
AGTTCTCCTGAGTGAGGCTCCATCCCATGGAGGGTGCAAGGCCCGTGTCGTGAGCAGCCGTCGCCGATGTGCGCTATCAG
AGAGTCGCCTTGTTTGCGAGTACAAGGTGAAGTCGGTGGTAAACTCCATCGAAGGCTAAATATGACCACGAGTCCGATAG
CGAACAAGTACCGTGAGGGAAAATTGAAAAGCACTTTGAAGAGAGAGCGAAACAGTGCGTGAAACCGCTCAGAGGCAAGC
AGATGGGGCCTCGAAGGCAGAGCCGCGAATTCAGCCGGTGGTCCGTGCGGTGGGTTGGGATTGGAGATCGCAAGACTCTG
CCTGGCTTACTTGGTGCGGCTACCGGTGCACTTTCGCGGCTTGTACGCCACCGCCGTTAAGGAGCGTCCGCCGGGTCTGC
GTGTGGAGCCTAACTGTCTTCGGGCAGTTGGTGTCTCACTGCGGGTCTGTGCGCGATCGCGCTTTAACCGGTCATGTCAG
CATGTGCCAGCGTTTGCGCTGGGTCAGCCGGCTCCGGTTGGGCTGTATGGGGATGTCGAGCTTGCTCGCCTCTTCTGCAC
CTGATGGACTTGTGTTGGCTTTCAGCGTGGTACATTGTGGATTCGGTGGCGAGTAGACGGCTGCCC

The **ITS-2** sequence (MG777531), 462 bp long:

AACGCACATTGCGGCTTTGGGTTGACTGAAGCCACGCCTGGTTGAGGGTCAGTTGAATAAACCATCACGGCTCATGCGTG
TAGCCGTGGATTGTCCGGATAACGTCCTTTGTGGCGTTAGCGGATCAAGTCTAGTCCGGATGTGGCTGGAAGTGAGCGTT
GGACTCGGACTGAAGCTTTTAATGCTTTGGCACTTGGTTGGGACGTTCGGCTTCTCGTGCACAAGCACCGCTGTGGCTTG
CTCGAGAGTGTCATCCAATTTATAAGTGTCAGAGTTTTCGGTCTAGTAGCAGAGTCTATGCCTACTAAAAGCGTGTATAT
CACATTCGCGTGCTTAACCCTTTCTTTTGGGGGTGTGTGTGTGTGTCCGATGCGACACATTATAACACCCCAATAAGAAA
TCCTTACTCATTCTTTTGACCTCAGCTCAGACGAGATTACCCGCTGAACTTAAGCATATCAA

The **COI** sequence (MG818723), 633 bp long:

TGAAGAGCTACAGTAGGAACTTCTCTTAGCATATTAATTCGATCCGAATTAAGACAACCAGGATTCCTTTTATCCGACGA
ACAACTCTATAATGTAACTGTAACAAGACATGCATTTGTAATAATTTTCTTTTTTGTTATACCCATTCTAATTGGAGGAT
TTGGTAACTGACTTATTCCCCTTATAATCGGGGCCCCAGACATAGCCTTTCCACGAATAAATAATCTAAGATTCTGGCTT
TTACCCCCATCATTTTTCCTAATCTCTACAAGAAGACTAAGAGAACAAGGAGCAGGAACAGGATGAACAGTCTATCCCCC

TCTAGCCCATTATTTTGCTCATAGAGGTCCAGCTGTCGATCTAACAATCTTCTCCCTTCACATTGCTGGAGTATCTTCAA
TTTTAGGAGCAGTAAATTTCATTTCAACTATTATCAATATGCGAACTCTTTCTATAAGTTTAGAAAACATGCCTTTATTT
GTATGATCAGTTCTCATCACAGCAGTGCTTCTTCTATTAGCACTACCCGTATTAGCAGGGGCAATTACCATACTATTACT
GGATCGAAATTTCAATACGTCATTCTTTGACCCAAGAGGTGGGGGAGACCCAATTCTATACCAACACTTATTC

The p-distances between haplotypes of all available *Hypsibius* species and *Borealibius zetlandicus* (Murray, 1907b) were as follows: 18S rRNA: from 0.3% (*H. convergens*, FJ435726 from Spain, and *H. pallidus*, HQ604945 from Italy) to 4.0% (*H. scabropygus* Cuénot, 1929, KC582831 from Austria), with the average distance of 1.9%; 28S rRNA: from 1.1% (*H. convergens*, FJ435771 from Spain) to 3.2% (*H. exemplaris*, MG800337), with the average distance of 2.4%; COI: from 17.3% (*H. convergens*, FJ435798 from Spain) to 22.8% (*H. exemplaris*, MG818724), with the average distance of 20.8%. The p-distance between the ITS-2 of *H. dujardini* and *H. exemplaris* is 12.6%. Full matrices with p-distances are provided in the Supplementary Material 2.

Etymology. Doyère (1840) named the species after Félix Dujardin (1801–1860), a distinguished French naturalist who also worked on tardigrades.

Hypsibius exemplaris sp. nov.

H. dujardini in: Gabriel & Goldstein (2007), Gabriel *et al.* (2007), Beltrán-Pardo *et al*. (2013), Tenlen *et al.* (2013), Smith and Jockusch (2014), Boothby *et al*. (2015), Gross & Mayer (2015), Arakawa *et al.* (2016), Bemm *et al*. (2016), Fernandez *et al*. (2016), Hering *et al*. (2016), Hyra *et al*. (2016), Koutsovoulos *et al*. (2016), Levin *et al*. (2016), Smith *et al*. (2016), Boothby *et al.* (2017), Erdmann *et al.* (2017), Gross *et al*. (2017), Smith *et al*. (2017), Yoshida *et al.* (2017), Gross *et al.* (2018);

H. cf. *dujardini* in: Kosztyła *et al*. (2016) and Stec *et al.* (2016).

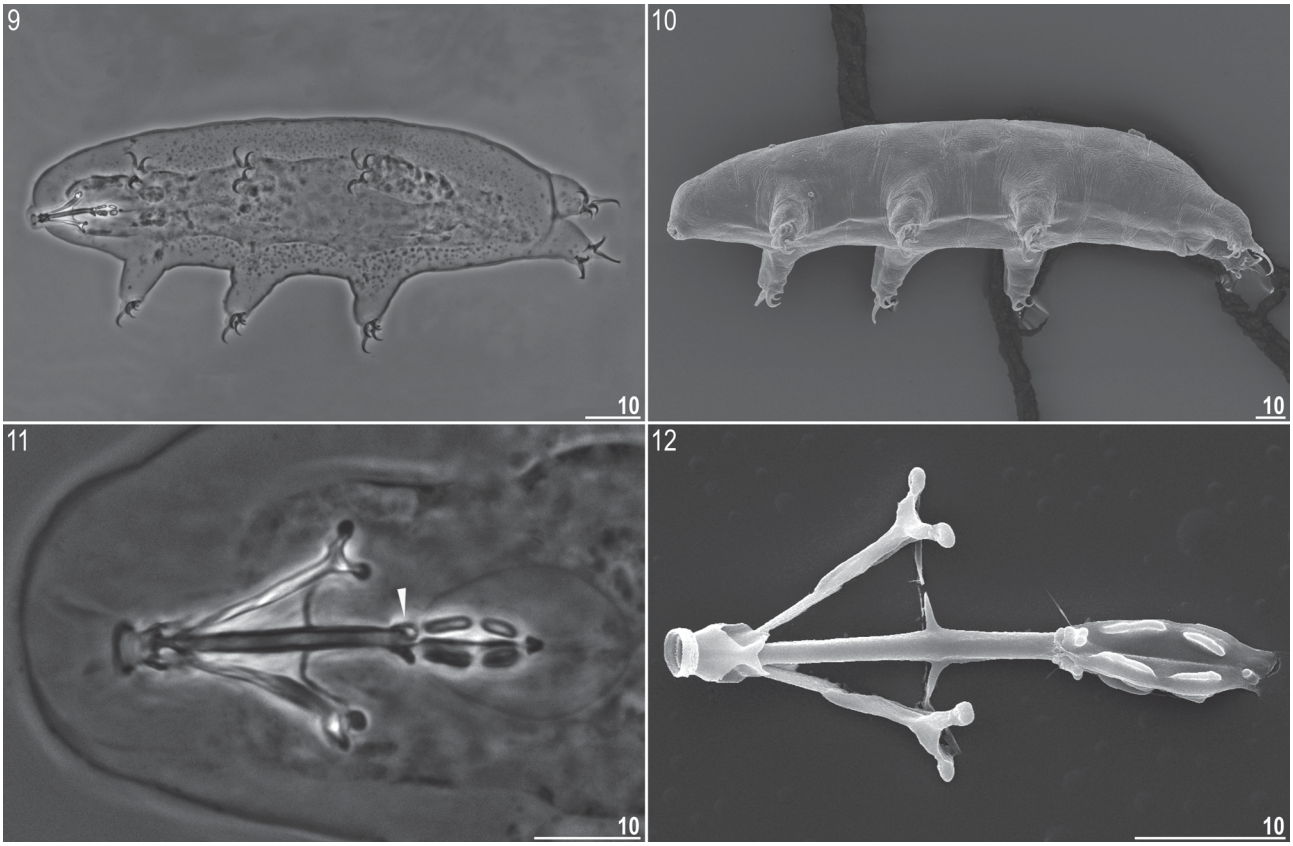
Locus typicus. 53°33’32’’N; 2°23’48’’W; 75 m asl: United Kingdom, England, Lancashire, Bolton, Darcy Lever; rotting leaves from a pond.

Material examined. Holotype and 64 paratypes from commercial isogenic culture (Sciento strain Z151) derived from a single female collected from Darcy Lever, Bolton, Lancashire by Robert McNuff (45 individuals on slides GB.003.01–10 and 20 paratypes on a SEM stub) deposited in the Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków, Poland. Paratypes mounted in Hoyer’s medium include 5 juveniles.

Integrative description. *Animals (see Table 5 for measurements):* Body elongated, transparent to whitish, covered with smooth cuticle, both under PCM and SEM (Figs 9–10). Eyes present in live animals, but prone to dissolution in Hoyer’s medium (Fig. 9). Buccal apparatus of the *Hypsibius* type (Figs 11–12). Mouth opening surrounded by a thin peribuccal ring without papulae or papillae. The oral cavity armature, visible only under SEM, consists of 3–4 rows of minute conical teeth located on the ring fold (Fig. 18, arrowhead). Two distinct porous areas on the lateral sides of the crown are visible in SEM only (Fig. 18, empty arrowhead). Stylet furcae of the *Hypsibius* type (Figs 11–12, 21). Pear-shaped muscle pharynx with eminent pharyngeal apophyses, two macroplacoids and a septulum (Figs 11, 24). Macroplacoid length sequence 2<1. In PCM, no constrictions are visible. Under SEM, both macroplacoids with slight constrictions: the first macroplacoid constricted anteriorly, the second—subterminally (Fig. 24, arrowheads). Claws of the *Hypsibius* type, with obvious accessory points on the primary branches (Figs 13–16). A clear septum dividing the claw into the basal and the branch portion; septum between the primary and the secondary branch typically less visible (Figs 13–14). In juveniles, claws have a uniform structure, without septa. Internal and anterior basal claws with thin, calyx-like trunks (Figs 13–16); anterior claws with evident pseudolunulae (Figs 14, 16, empty arrowheads). Between the posterior and the anterior claw a sigmoidal longitudinal bar is present. The bar is typically connected with the posterior claw base (Figs 14, 16, arrowheads). Cuticular bars on legs I–III absent.

Eggs: Roundish and smooth, deposited in exuviae (up to thirty six per clutch observed in the culture).

Molecular markers: The sequences for all four DNA markers and four specimens (isogenophores) were of a very good quality. All markers were represented by a single haplotype:



FIGURES 9–12. *Hypsibius exemplaris* **sp. nov.**: **9**—adult habitus (ventrolateral view, PCM, holotype); **10**—adult habitus (lateral view, SEM, paratype); **11**—bucco-pharyngeal apparatus, the arrowhead indicates large pharyngeal apophyses (PCM, paratype); **12**—bucco-pharyngeal apparatus (SEM, paratype). All scale bars in μm.

The **18S rRNA** sequence (MG800327, same as HQ604943), 1,038 bp long:

TCCTAGATCGTACAGTTTACATGGATAACTGTGGTAATTCTAGAGCTAATACATGCAACCAGTCCGTTCCCTCGTGGAGC
GGACGCAGTTATTTGCCCAAGACCAATCCGGCCCTCGGGTCGGTCAATTGGTGACTCTGAATAACCGAAGCGGAGCGCAT
GATCTCGTATCGGCGCCAGATCTTTCAAGTGTCTGACTTATCAGCTTGTTGTTAGGTTATGTTCCTAACAAGGCTTTTAC
GGGTAACGGAGTGTCAGGGCCCGACACCGGAGAGGGAGCCTGAGAAACGGCTACCACATCCAAGGAAGGCAGCAGGCGCG
CAAATTACCCACTCCCGGCACGGGGAGGTAGTGACGAAAAATAACGATGCGAGAGCTTTTAGCTTCTCGTAATCGGAATG
GGTACACTTTAAATCCTTTAACGAGGATCTATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATA
GCGTATATTAAAGTTGCTGCGGTTAAAAAGCTCGTAGTTGGATCTGGGTAGTCGATGGACGGTTCTTCGTAAGAAGATAC
TGCCCGTTCGGCACCACAGCCCGGCCATGTCTTGCATGCTCTTCACTGAGTGTGCTTGGCGACCGGAACGTTTACTTTGA
AAAAATTAGAGTGCTCAAAGCAGGCGTTAAGCCTTGTATAATGGTGCATGGGATAATGGAATAAGATTTTTGGCTTGTTC
TGTTGGTCTTAGAGTCAGAAGTAATGATAAATAGGAACAGACGGGGGCATTCGTATTGCGGCGTTAGAGGTGAAATTCTT
GGATCGTCGCAAGACGCACTACTGCGAAAGCATTTGCCAAGAATGTTTTCATTAATCAAGAACGAAAGTTAGAGGTTCGA
AGGCGATCAGATACCGCCCTAGTTCTAACCATAAACGATGCCAACCAGCGATCCGTCGGTGTTTATTTGATGACTCGACG
GGCAGCTTCCGGGAAACCAAAGTGCTTAGGTTCCGGGGGAAGTATGGTTGCAAAGCTGAAACTTAAAGGAATGACGAA

The **28S rRNA** sequence (MG800337), 814 bp long:

TTAAGCATATTACTAAGCGGAGGAAAAGAAACCAACGGGGATTCCCATAGTAACTGCGAGTGAAAGGGGAAAAGCCCAGC
GCCGAATCCTGCCGCTGGAGACGGTGGCAGGAACTGTGGCGTGAAGATGGTATGTACCGGTGTGGCTCGCTCGCGTAAGT
TCTCCTGAGTGAGGCTCCATCCCATGGAGGGTGCAAGGCCCGTGTCGTGAGCAGCCGTCGCCGGTGTGTGCTATCAGAGA
GTCGCCTTGTTTGCGAGTACAAGGTGAAGTCGGTGGTAAACTCCATCGAAGGCTAAATATGACCACGAGTCCGATAGCGA
ACAAGTACCGTGAGGGAAAATTGAAAAGCACTTTGAAGAGAGAGCGAAACAGTGCGTGAAACCGCTCAGAGGCAAGCAGA
TGGGGCCTCGAAGGCAGAGCCGCGAATTCAGCCGGTGGTCCGTGCGGTGTGTCGGGATGGGAGATCGCAAGACTCTGCCT
GGCTTACTGGTGCGGCTGCCGGTGCACTTTCGCGGCTTGTACGCCACCGCCGTTAAGGAGCGTCCACCGGGCCTGCATGT
GGAGCCTAGCTGTCTTCGGGCAGTTGGTGTCTCACGGCGGGTCTGTGTGCGATCGCGCTTTAACCGGTCATGTCAGCATG
TGTCAGCGTTTGCGCTGGGTCAGCCGGCTCCGGTTGGGCTGTATGGGGATGACGAGCTTGCTCGGCTCTCCTGCACCTGA

TGGACTCGTGCGGGCTTTCAGCGTGGCACATTGTGGATTCGGTGGCGAGTAGACAGCTGCCCATCTACCCGTCTTGAACA
CGGGAACAAAGGAA

The **ITS-2** sequence (MG800336), 441 bp long:

ACGCACATTGCGGCTTTGGGTTGACTGAAGCCACGCCTGGTTGAGGGTCAGTTGAATAAACCATCACGGTTCATGCGTGT
AACTGTGGATTGTCCGGATAACGCTCCTTCACCGGAGCGTTAGCGGATCAAGTCTAGTCCGGATGTGGCTGGAGGTGAGC
GTTGGACTTGGACCGAAGCTTACGGGCTTTGGCGCGGTTGGGACGTTCGGCTTCTCGTGCACATGCACCGCTGTTGCATG
CTCGAGAGTGTCATCCAACGCAGCGTCAGAGTCTTTCGGTTTAGCAGCAGAGTCTATGCTTGATTTTCGGCGTGCTTTTC
ACATTCGCGTGGTAAAACAACTCGGTGGGGTGACCCCGTCGCGGTCACCACCGAAAAATCTTTACTCATTCTTTTGACCT
CCGCTCAGACGAGATTACCCGCTGAACTTAAGCATATCAAA

The **COI** sequence (MG818724, same as KU513418) 794 bp long:

TATCTGAAGAGCAACTGTAGGAACCTCCCTAAGCATACTAATTCGTTCTGAGCTTAGCCAACCAGGAAGCTTATTAGGAG
ACGAACAAATTTACAACGTAACTGTTACCAGACATGCATTTATTATAATTTTCTTCTTTGTAATACCTATTCTAATTGGA
GGATTCGGAAACTGATTAATTCCTCTTATAATTGGGGCTCCAGACATAGCTTTCCCTCGCTTAAACAATCTTAGGTTCTG
ACTTCTACCACCGTCTTTCTTTCTTATTACTTCTAGCACCGTCAGAGAACAGGGGGCCGGTACAGGGTGAACCGTATACC
CTCCTCTGGCACACAATTTTGCACATAGAGGTCCAGCAGTGGATCTGACAATTTTTTCCCTTCACCTAGCCGGAGTGTCA
TCTATTTTAGGGGCAACAAACTTTATTTCAACAATTATTAATATGCGCACATCCTCTATAATACTGGAAAGTATACCCCT
CTTTGTTTGATCTGTTCTAATCACGGCAGTTTTACTGCTTTTAGCCCTACCTGTTCTAGCAGGGGCCATTACCATATTGC
TACTAGATCGTAACTTTAACACATCCTTCTTCGACCCTAGAGGAGGAGGAGACCCGATTCTCTATCAACACTTATTTTGG
TTCTTCGGACACCCAGAAGTATATATTCTGATTCTTCCCGGATTCGGAATCATTTCTCAAATTATTGCCCACTATAGGGG
AAAGCATCTAGTATTCGGACATTTAGGGATAGTATACGCTATAAGAACAATTGGTCTCCTAGGGTTTATTGTAT

The p-distances between haplotypes of all available *Hypsibius* species and *Borealibius zetlandicus* (Murray, 1907b) were as follows: 18S rRNA: from 1.5% (*B. zetlandicus*, FJ184601 from Italy) to 4.0% (*H. scabropygus* Cuénot, 1929, KC582831 from Austria), with the average distance of 2.5%; 28S rRNA: from 3.0% (*H. convergens*, FJ435771 from Spain) to 3.6% (*H. klebelsbergi* Mihelčič, 1959, KC582835 from Austria), with the average distance of 3.3%; COI: from 22.5% (*B. zetlandicus*, FJ184601 from Italy) to 24.7% (*H. convergens*, FJ435798 from Spain), with the average distance of 23.3%. Full matrices with p-distances are provided in the Supplementary Material 2.

Etymology. From Latin *exemplaris* = exemplary, model. The name refers to the wide use of the species as a laboratory model for various types of scientific studies.

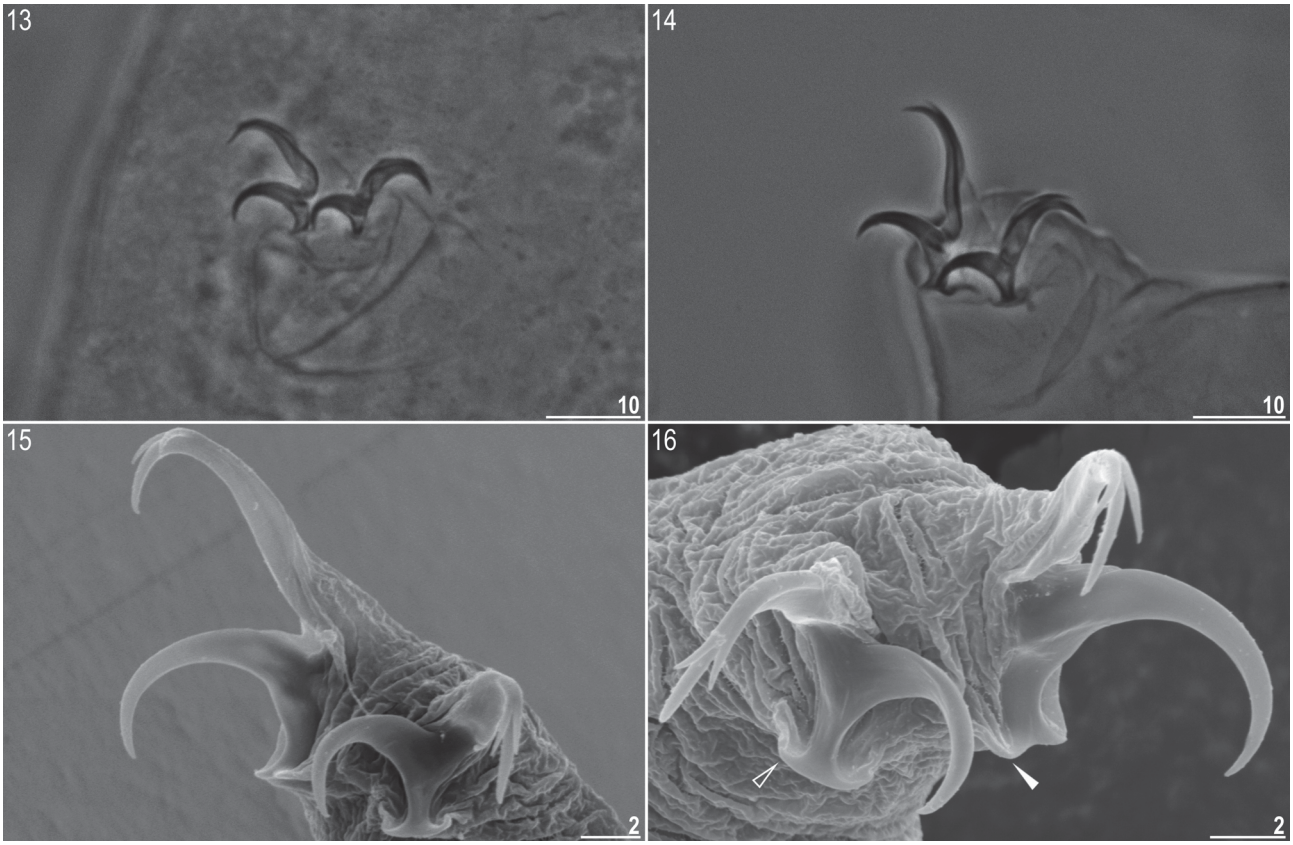
Differential diagnoses. *H. dujardini* is the nominal taxon for a group of *Hypsibius* species (*i.e*. the *dujardini* group) that is characterised by smooth cuticle, and two macroplacoids and septulum in the pharynx. The general similarities between *H. dujardini* and *H. convergens* (Fig. 25) means these are often considered to form a large species complex. However, there is insufficient molecular evidence to verify whether the *H. dujardini* and *H. convergens* complexes are immediate relatives or they represent different clades. Nevertheless, the two species groups, despite obvious similarities, seem to be morphologically divergent in the buccal apparatus morphology. Whereas species of the *H. dujardini* complex have a septulum in the pharynx (Figs 3–4 and 11–12), this structure is absent in the *H. convergens* complex (Fig. 26). Although some individuals of the *H. convergens* complex have a fine roundish thickening posterior to the second macroplacoid, it cannot be considered a proper septulum due to its rudimental size, whereas a fully developed septulum is always evident in species of the *H. dujardini* complex. Moreover, species in the *convergens* group have more robust claws in comparison with members of the *dujardini* complex (compare Figs 5–6, 13–14 and Figs 27–29). Nonetheless, an integrative redescription of *H. convergens* from the *locus typicus* is urgently required to clarify the taxonomic status of the two complexes.

Up to now, seven species have been described in the *H. dujardini* complex: *Hypsibius conwentzii* Kaczmarek *et al*., 2018, *H. heardensis* Miller *et al.*, 2005, *H. pallidoides* Pilato *et al*., 2011, *H. septulatus* Pilato *et al*., 2004, *H. seychellensis* Pilato *et al*., 2006, *H. valentinae* Pilato *et al*., 2012, and *H. exemplaris* **sp. nov.** presented in this work. Nevertheless, *H. dujardini* can be easily distinguished from the abovementioned species and it differs specifically from:

Hypsibius conwentzii, recently described from maritime Antarctic (Kaczmarek *et al.*, 2018), by a shorter septulum (0.7–1.7 µm [*3.3–6.5%*] in *H. dujardini vs* 1.8–2.6 µm [*7.6–10.2%*] in *H. conwentzii*), and by the absence of cuticular bars on legs I–III (bars at internal claws I–III present in *H. conwentzii*).

***Hypsibius exemplaris* sp. nov.**, described from north-west England and maintained in laboratories throughout the world, by body shape (stubby in *H. dujardini vs* elongated in *H. exemplaris*), a more anterior stylet support insertion

point (*57.2–64.2%* in *H. dujardini vs 65.6–68.4%* in *H. exemplaris*), a slightly different macroplacoid shape (more robust in *H. dujardini vs* prolate in *H. exemplaris*; compare Figs 3–4 and 11–12, respectively), and by claw IV morphology (broad base trunks in *H. dujardini vs* calyx-like and slender in *H. exemplaris*; compare Figs 5–8 and 13–16, respectively).



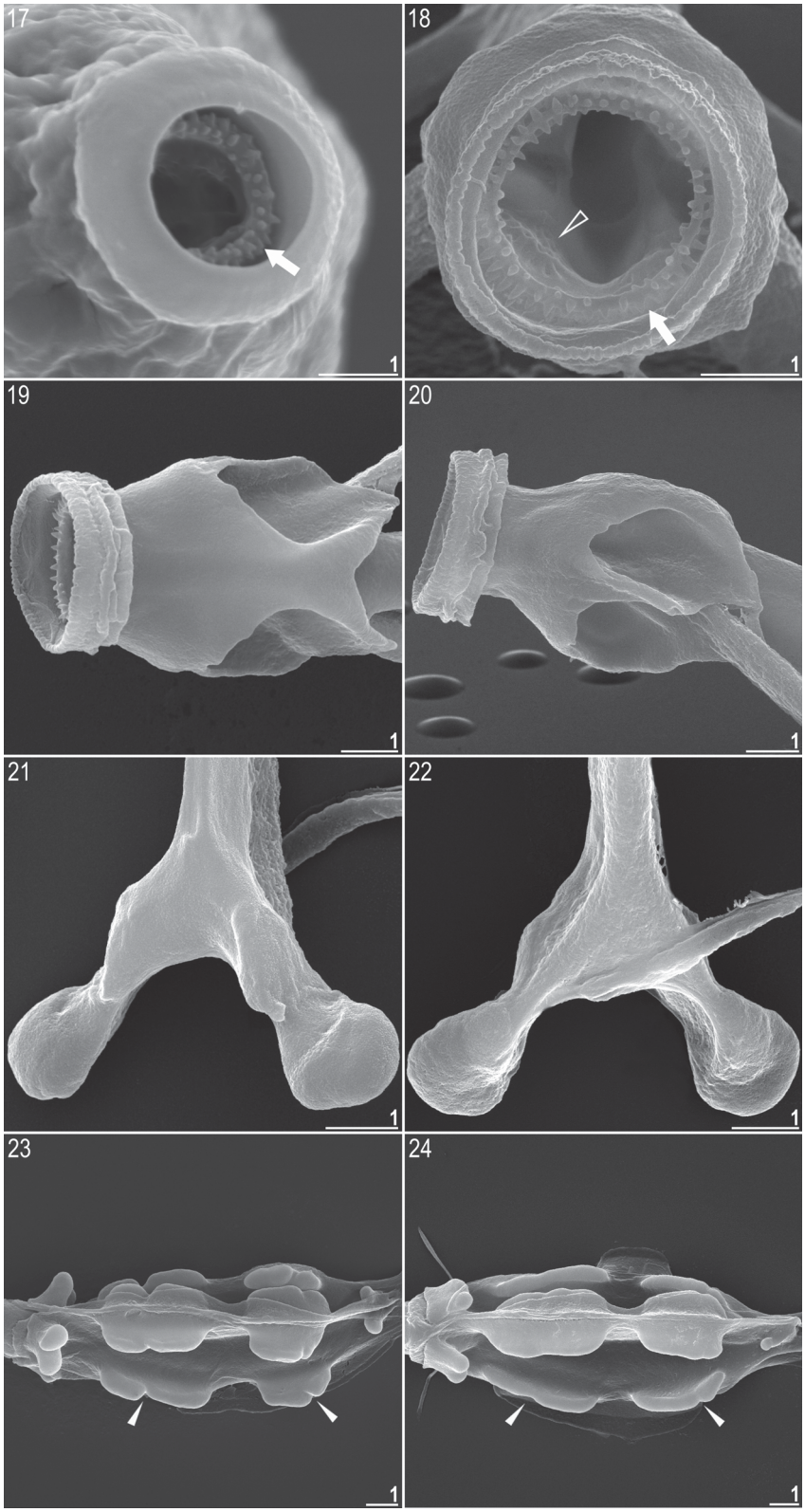
FIGURES 13–16. *Hypsibius exemplaris* **sp. nov.**, claws: **13**—claws III (PCM, holotype); **14**—claws IV, arrowhead points longitudinal bar at the posterior claw basis, and empty arrowhead indicates pseudolunula at the anterior claw basis (PCM, paratype); **15**—claws III (SEM, paratype); **16**—claws IV, arrowhead points longitudinal bar at the posterior claw basis, and empty arrowhead indicates pseudolunula at the anterior claw basis (SEM, paratype). All scale bars in μm.

Hypsibius heardensis, known from its *locus typicus* on Heard Island, and from Macquarie Island in sub-Anarctic (Miller *et al.*, 2005), by the presence of eyes (present in live *H. dujardini vs* absent in *H. heardensis*, although the original description does not state whether the existence of eyes was examined before or after mounting), and the absence of bars on legs I–III bases (bars at internal claw bases present in *H. heardensis*). According to Miller *et al*. (2005), *H. dujardini* is supposed to have a "large" septulum whereas *H. heardensis*—has a "small" septulum, and they use this trait to differentiate the two taxa. However, the present study, in which the dimensions of the septulum in *H. dujardini sensu stricto* are provided for the first time, shows that length ranges of this structure overlap in the two species (0.7–1.7 μm in *H. dujardini vs ca.* 1.0 μm in *H. heardensis*) and thus it cannot be used here as a differentiating trait.

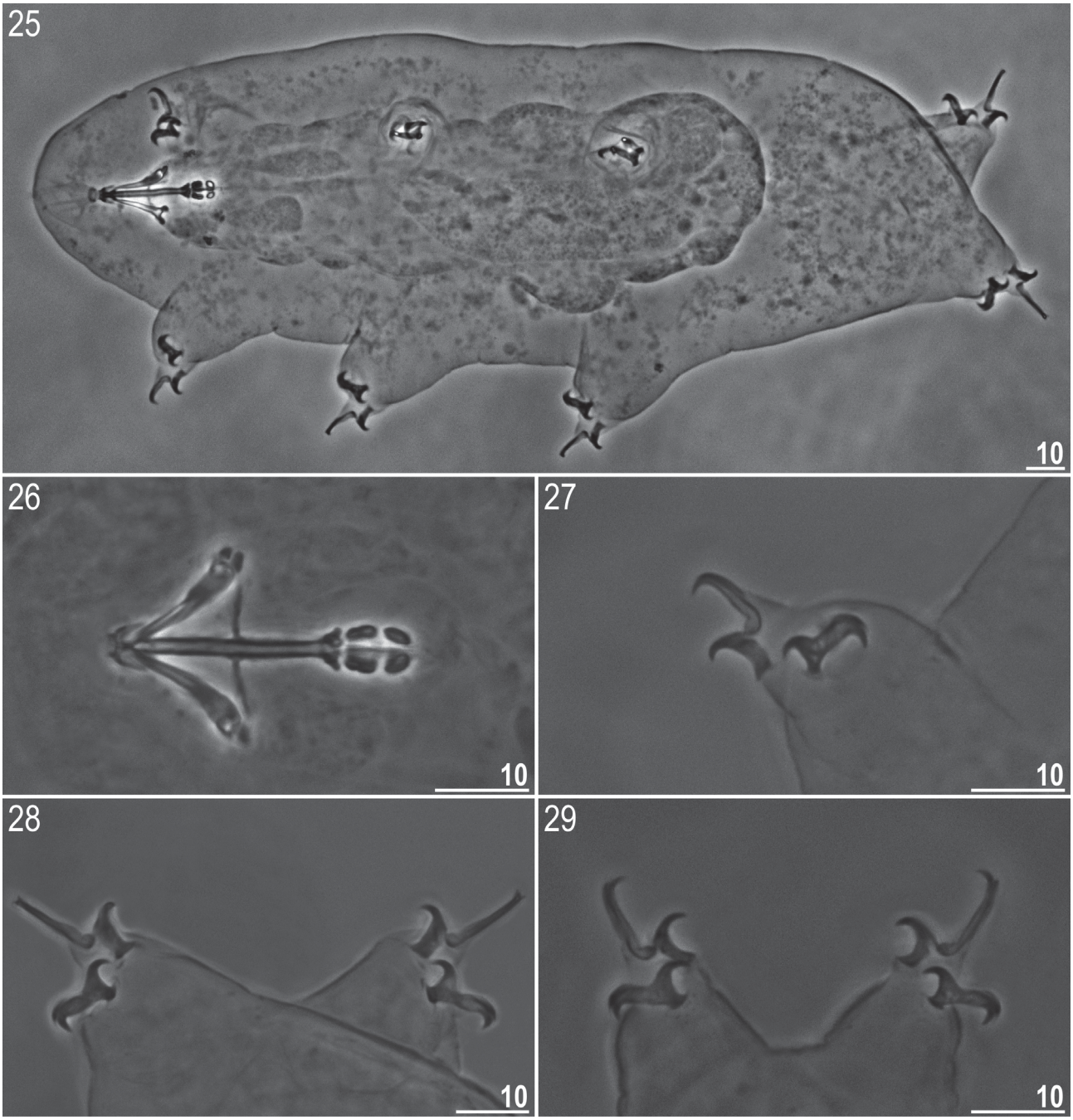
Hypsibius pallidoides, recorded only from the type locality in southern Ukraine (Pilato *et al.*, 2011), by stylet supports inserted in a more caudal position (*57.2–64.2%* in *H. dujardini vs 54.2–55.5%* in *H. pallidoides*), shorter external and posterior claw primary branches (5.9–11.5 μm and 6.5–14.0 μm in *H. dujardini vs* 12.7–14.6 μm and 17.7–18.6 μm in *H. pallidoides*; excluding the lengths of external primary branches I, as they were not presented in the description of *H. pallidoides*) also manifested as lower *pt* values (*32.4–47.4%* and *40.9–56.3%* in *H. dujardini vs 54.3–57.0%* and *68.6–72.1%* in *H. pallidoides*), and by the presence of bars on legs IV (absent in *H. pallidoides*). Pilato *et al*. (2011) stated that the buccal tube width in *H. dujardini* gradually increases towards its posterior end. However, the present study showed unambiguously that *H. dujardini s.s.* has the buccal tube of equal width on its entire length (see Figs 3–4), as does *H. pallidoides*. Thus, buccal tube shape is not discriminant between the two species.

TABLE 5. Measurements [in µm] of selected morphological structures of type individuals of *Hypsibius exemplaris* **sp. nov.** mounted in Hoyer's medium. N—number of specimens/structures measured, RANGE refers to the smallest and the largest structure among all measured specimens; SD—standard deviation.

CHARACTER	N	RANGE		MEAN		SD		Holotype	
		µm	*pt*	µm	*pt*	µm	*pt*	µm	*pt*
Body length	30	112 – 293	*602 – 1028*	232	*882*	36	*83*	250	*962*
Buccal tube									
Buccal tube length	30	18.6 – 29.7	–	26.1	–	2.2	–	26.0	–
Stylet support insertion point	30	12.2 – 19.7	*65.6 – 68.4*	17.5	*66.9*	1.5	*0.9*	17.1	*65.8*
Buccal tube external width	30	1.4 – 2.5	*7.4 – 9.4*	2.2	*8.6*	0.2	*0.5*	2.3	*8.8*
Buccal tube internal width	30	0.7 – 1.4	*3.2 – 5.3*	1.1	*4.1*	0.2	*0.5*	1.2	*4.6*
Placoid lengths									
Macroplacoid 1	30	2.9 – 5.4	*15.6 – 19.5*	4.6	*17.5*	0.5	*1.1*	4.2	*16.2*
Macroplacoid 2	30	2.5 – 4.5	*12.1 – 16.8*	3.6	*13.9*	0.5	*1.1*	3.6	*13.8*
Septulum	30	1.3 – 2.0	*4.9 – 7.5*	1.6	*6.2*	0.2	*0.6*	1.6	*6.2*
Macroplacoid row	30	6.4 – 10.5	*32.3 – 38.9*	9.3	*35.4*	0.9	*1.5*	9.0	*34.6*
Claw 1 lengths									
External base	15	2.6 – 4.2	*10.3 – 14.9*	3.2	*12.2*	0.4	*1.3*	3.7	*14.2*
External primary branch	10	8.9 – 11.7	*33.3 – 41.5*	10.0	*37.9*	0.9	*2.3*	10.5	*40.4*
External secondary branch	10	6.5 – 8.9	*25.0 – 31.8*	7.3	*27.8*	0.8	*2.4*	6.5	*25.0*
Internal base	16	2.2 – 3.1	*8.8 – 11.9*	2.7	*10.2*	0.3	*0.8*	3.1	*11.9*
Internal primary branch	4	7.8 – 9.3	*27.9 – 31.7*	8.5	*29.8*	0.7	*1.6*	?	*?*
Internal secondary branch	12	4.4 – 6.1	*16.8 – 22.3*	5.2	*19.6*	0.6	*1.8*	4.8	*18.5*
Claw 2 lengths									
External base	19	2.5 – 4.0	*9.5 – 15.9*	3.3	*12.7*	0.5	*1.6*	3.4	*13.1*
External primary branch	17	9.3 – 13.2	*37.5 – 45.7*	10.8	*40.9*	1.2	*2.8*	10.3	*39.6*
External secondary branch	17	6.7 – 8.2	*24.5 – 31.5*	7.4	*28.2*	0.5	*1.9*	8.2	*31.5*
Internal base	23	2.1 – 3.1	*8.9 – 12.8*	2.8	*10.7*	0.3	*0.9*	2.6	*10.0*
Internal primary branch	8	7.3 – 10.0	*26.6 – 33.7*	8.1	*29.3*	0.9	*2.2*	7.3	*28.1*
Internal secondary branch	19	3.6 – 7.1	*18.1 – 25.6*	5.6	*21.6*	0.9	*2.1*	5.7	*21.9*
Claw 3 lengths									
External base	22	2.6 – 4.1	*10.6 – 16.0*	3.4	*13.1*	0.4	*1.5*	3.2	*12.3*
External primary branch	15	9.2 – 11.7	*35.1 – 43.4*	10.6	*39.7*	0.8	*2.4*	11.1	*42.7*
External secondary branch	18	6.4 – 9.4	*25.5 – 32.3*	7.7	*28.9*	0.8	*2.0*	8.4	*32.3*
Internal base	21	1.7 – 3.4	*8.4 – 12.4*	2.7	*10.4*	0.4	*1.2*	2.8	*10.8*
Internal primary branch	11	6.7 – 9.1	*24.5 – 32.1*	7.7	*28.4*	0.9	*2.6*	7.8	*30.0*
Internal secondary branch	20	5.1 – 7.6	*19.8 – 25.6*	6.0	*22.6*	0.7	*1.9*	5.8	*22.3*
Claw 4 lengths									
Anterior base	22	1.6 – 4.0	*8.6 – 13.5*	3.0	*11.4*	0.5	*1.3*	3.3	*12.7*
Anterior primary branch	10	7.6 – 11.0	*30.4 – 37.2*	9.1	*34.4*	1.1	*2.1*	8.9	*34.2*
Anterior secondary branch	14	5.0 – 7.6	*20.0 – 26.0*	6.1	*22.7*	0.7	*1.8*	6.1	*23.5*
Posterior base	27	3.3 – 5.1	*12.2 – 17.7*	4.0	*15.2*	0.4	*1.3*	4.6	*17.7*
Posterior primary branch	18	11.3 – 15.7	*45.0 – 54.7*	13.1	*50.2*	1.2	*3.1*	14.2	*54.6*
Posterior secondary branch	22	5.6 – 9.9	*27.1 – 35.1*	7.9	*30.6*	0.9	*2.0*	?	*?*



FIGURES 17–24. Details of the bucco-pharyngeal apparatus of the *Hypsibius* type (in SEM): **17**—peribuccal ring and the oral cavity armature of *H. dujardini*, the arrow indicates the row of conical teeth located on the ring fold; **18**—oral cavity armature of *H. exemplaris* **sp. nov.**, the arrow indicates the row of conical teeth located on the ring fold whereas the empty arrowhead indicates the porous area on the lateral wall of the cavity; **19**—the buccal crown and the dorsal apophyses for insertion of stylet muscles (AISM) of *H. exemplaris*; **20**—the buccal crown and both dorsal and ventral apophyses for insertion of stylet muscles (AISM) of *H. exemplaris* **sp. nov.** in lateral view; **21**—furca of *H. exemplaris* **sp. nov.**, external side; **22**—furca of *H. dujardini*, internal side with the stylet support; **23**—pharynx of *H. dujardini*, arrowheads point out evident macroplacoid constrictions; **24**—pharynx of *H. exemplaris* **sp. nov.**, arrowheads point out subtle macroplacoid constrictions. All scale bars in μm.



FIGURES 25–29. *Hypsibius* cf. *convergens* (Urbanowicz, 1925) from Poland, seen in PCM: **25**—habitus, ventral view; **26**—bucco-pharyngeal apparatus; **27**—claws I; **28**—claws IV; *Hypsibius pallidus* Thulin, 1911 from Poland, seen in PCM: **29**—claws IV. All scale bars in μm.

Hypsibius septulatus, reported only from its *locus typicus* in Tierra de Fuego (Pilato *et al.*, 2004), by the dorsal cuticle surface (smooth in *H. dujardini vs* with numerous undulations in *H. septulatus*), by the lengths of external and posterior primary branches (5.9–14.0 μm in *H. dujardini vs* 15.6–17.4 μm in *H. septulatus*), internal + anterior primary branches (4.6–9.4 μm in *H. dujardini vs* 10.4–11.0 μm in *H. septulatus*; excluding the lengths of external primary branches I, as they were not presented in the description of *H. septulatus*), also manifested as lower *pt* values (*32.4–56.3%* and *24.0–36.1%* in *H. dujardini vs 63.7–68.8%* and *42.4–44.5%* in *H. septulatus*), and by the presence of bars on legs I–III (absent in *H. dujardini vs* bars at both external and internal claw bases present in *H. septulatus*).

Hypsibius seychellensis, recorded exclusively from the Seychelles Archipelago (Pilato *et al.*, 2006), by the

second macroplacoid shape (ovoid in *H. dujardini vs* granular in *H. seychellensis*), relatively wider external buccal tube diameter (*pt=6.9–10.2%* in *H. dujardini vs 6.3–6.4%* in *H. seychellensis*), and by relatively shorter septulum (*pt=3.3–6.5%* in *H. dujardini vs 7.1–8.1%* in *H. seychellensis*). Since other discriminative morphometric traits given by Pilato *et al*. (2006) fall within the variability range of *H. dujardini*, they are invalid.

Hypsibius valentinae, known from central and northern Belarus (Pilato *et al.*, 2012), only by shorter external and posterior primary branches (5.9–14.0 µm in *H. dujardini vs* 14.5–17.2 µm in *H. valentinae*), and by and internal and anterior primary branches (4.6–9.4 µm in *H. dujardini vs* 9.3–11.5 µm in *H. valentinae*). Pseudolunulae under internal and anterior claws are present in both species (these structures were defined as "lunulae" in Pilato *et al*. 2012 but the term "pseudolunula" is more appropriate to differentiate the weak cuticular outlines present under claws in hypsibiids and isohypsibiids from well-defined lunulae connected with the claw by a peduncle observed in macrobiotids and eohypsibiids; see Gąsiorek *et al*. 2017b).

It should be noted that specimens, from undefined localities, classified by Pilato *et al.* (2006a, 2011, 2012) as *H. dujardini* and used by them in their works for comparisons with various *dujardini* group species differ substantially from the neotypic population of *H. dujardini* presented here. Therefore, those individuals most likely represent a new species (see also Discussion below). Considering the low number of meaningful traits within the *dujardini* complex, descriptions of new species within this group should be supported by molecular data. More comprehensive morphometric datasets for *H. heardensis*, *H. seychellensis*, and *H. valentinae* (including both larger numbers of measured structures and specimens) could also provide novel traits needed for species delineation within the *dujardini* complex.

Hypsibius exemplaris **sp. nov.** has to be compared with the same species, and it differs specifically from:

Hypsibius conwentzii, by a more caudal stylet support insertion point (*65.6–68.4%* in *H. exemplaris* **sp. nov.** *vs 58.6–62.4%* in *H. conwentzii*), a relatively shorter septulum (*4.9–7.5%* in *H. exemplaris* **sp. nov.** *vs 7.6–10.2%* in *H. conwentzii*), and by the absence of cuticular bars on legs I–III (bars at internal claws I–III present in *H. conwentzii*).

Hypsibius dujardini—please see the differential diagnosis for *H. dujardini* above.

Hypsibius heardensis, by a more caudal stylet support insertion point (*65.6–68.4%* in *H. exemplaris* **sp. nov.** *vs 56.0–63.0%* in *H. heardensis*), the presence of eyes (present in live *H. exemplaris* **sp. nov.** *vs* absent in *H. heardensis*, although the original description does not state whether the existence of eyes was examined before or after mounting), and by the presence of bars on legs I–III (absent in *H. exemplaris vs* bars at internal claw bases present in *H. heardensis*).

Hypsibius pallidoides, by a more caudal stylet support insertion point (*65.6–68.4%* in *H. exemplaris* **sp. nov.** *vs 54.2–55.5%* in *H. pallidoides*), shorter posterior primary branches (11.3–15.7 µm in *H. exemplaris* **sp. nov.** *vs* 17.7–18.6 µm in *H. pallidoides*), also manifested as lower *pt* values (*45.0–54.7%* in *H. exemplaris* **sp. nov.** *vs 68.6–72.1%* in *H. pallidoides*), and by the presence of bars on legs IV (bars between claw IV bases present in *H. exemplaris* **sp. nov.** *vs* bars absent in *H. pallidoides*).

Hypsibius septulatus, by body shape (elongated in *H. exemplaris* **sp. nov.** *vs* stubby in *H. septulatus*), the dorsal cuticle surface (smooth in *H. exemplaris* **sp. nov.** *vs* with numerous undulations in *H. septulatus*), a relatively shorter macroplacoid 1 (*pt=15.6–19.5%* in *H. exemplaris* **sp. nov.** *vs 21.2%* in *H. septulatus*), shorter external, internal and posterior primary branches (8.9–15.7 µm and 6.7–10.0 µm in *H. exemplaris* **sp. nov.** *vs* 15.6–17.4 µm and 10.4–10.9 µm in *H. septulatus*), also manifested as lower *pt* values (*33.3–54.7%* and *24.5–33.7%* in *H. exemplaris* **sp. nov.** *vs 63.7–68.8%* and *42.4–44.5%* in *H. septulatus*), and the presence of bars on legs I–III (absent in *H. exemplaris* **sp. nov.** *vs* bars at both external and internal claw bases present in *H. septulatus*).

Hypsibius seychellensis, by body shape (elongated in *H. exemplaris* **sp. nov.** *vs* stubby in *H. seychellensis*), a more caudal stylet support insertion point (*65.6–68.4%* in *H. exemplaris* **sp. nov.** *vs 62.3–63.7%* in *H. seychellensis*), the second macroplacoid shape (elongated in *H. exemplaris* **sp. nov.** *vs* granular in *H. seychellensis*), and by relatively wider external buccal tube diameter (*pt=7.4–9.4%* in *H. exemplaris* **sp. nov.** *vs 6.3–6.4%* in *H. seychellensis*).

Hypsibius valentinae, by a more caudal stylet support insertion point (*65.6–68.4%* in *H. exemplaris* **sp. nov.** *vs 61.3–62.5%* in *H. valentinae*), a relatively shorter macroplacoid 1 (*pt=15.6–19.5%* in *H. exemplaris* **sp. nov.** *vs* 20*.6–22.1%* in *H. valentinae*), and by shorter external and posterior primary branches (8.9–13.2 µm and 11.3–15.7 µm in *H. exemplaris* **sp. nov.** *vs* 14.5–16.5 µm and 17.1–17.2 µm in *H. valentinae*), also manifested as lower *pt* values (*33.3–45.7%* and *45.0–54.7%* in *H. exemplaris* **sp. nov.** *vs 53.5–60.9%* and *63.1–63.2%* in *H. valentinae*).

The differential diagnoses presented above clearly show that species within the *H. dujardini* complex are distinguished almost exclusively by morphometric traits. This explicitly underlines the need for careful measurements of a considerable number of individuals in order to secure the reliability of species delineation based on phenotypic traits (see Stec *et al.* 2016 for recommendations on sample size in tardigrade morphometry).

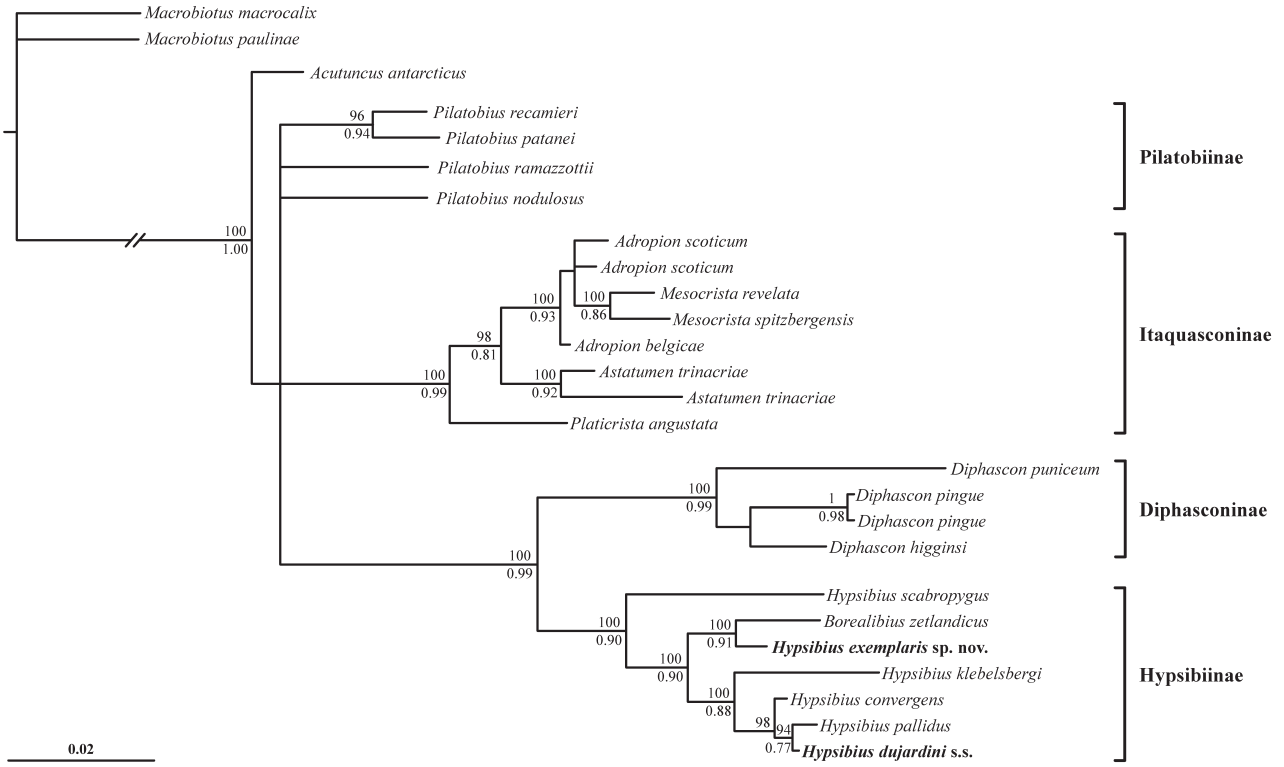


FIGURE 30. A Bayesian phylogenetic tree of the family Hypsibiidae based on 18S rRNA sequences, with two *Macrobiotus* spp. as an outgroup. Bayesian posterior probability values are given above tree branches whereas ML support values are below branches. The scale refers to the Bayesian tree.

Phylogeny. Both the BI and the ML 18S rRNA phylogenetic trees share identical topology (Fig. 30). Out of four subfamilies, three were highly supported (Itaquasconinae and Diphasconinae + Hypsibiinae clade, BI=1.00, ML=0.99), although remaining in polytomy. In contrast, *Pilatobius* spp. did not cluster in a monophyletic clade that would represent the subfamily Pilatobiinae.

Within the Hypsibiinae, *Hypsibius* is both polyphyletic and paraphyletic with respect to *Borealibius* (Fig. 30). The ML COI phylogenetic tree, being more suitable for inference at lower taxonomic levels, confirms the polyphyly of the genus (Fig. 31), placing the potential *Hypsibius s.s*. (*i.e.* the *convergens* and *dujardini* groups) as the sister clade to *Borealibius*, whereas the morphologically distant *H. klebelsbergi* is a sister taxon to this group.

Taxonomic status of *Ramazzottius conifer* (Mihelčič, 1938) comb. nov.

Phylum: Tardigrada Doyère, 1840
Class: Eutardigrada Richters, 1926
Order: Parachela Schuster, Nelson, Grigarick and Christenberry, 1980
Superfamily: Hypsibioidea Pilato, 1969 (in Marley et al. 2011)
Family: Ramazzottiidae Sands, McInnes, Marley, Goodall-Copestake, Convey & Linse, 2008
Genus: *Ramazzottius* Binda & Pilato, 1986
Material examined: Two specimens and three eggs on two slides deposited in the Institute of Zoology and Biomedical Research, Jagiellonian University, Kraków, Poland.

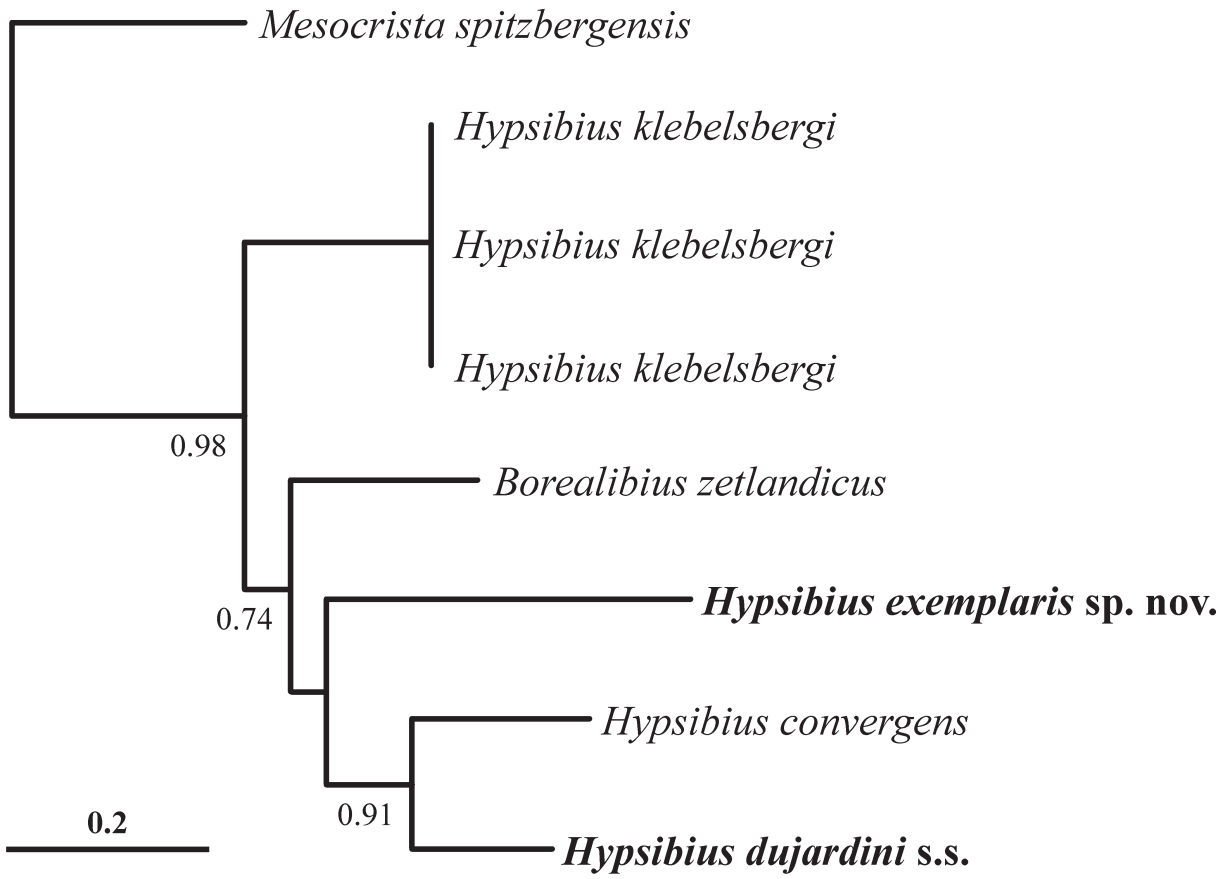


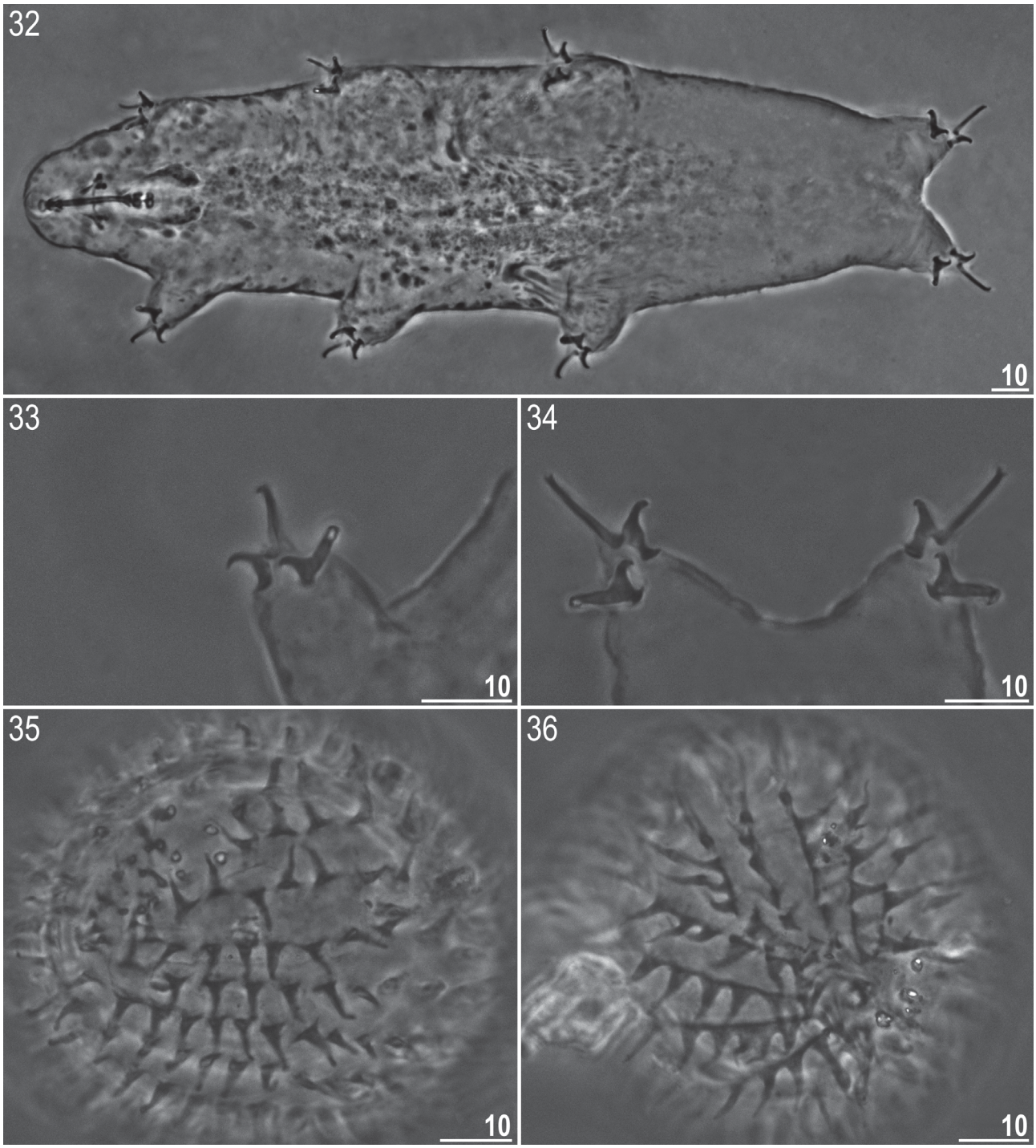
FIGURE 31. An ML COI-based phylogenetic tree of the subfamily Hypsibiinae with; *Mesocrista spitzbergensis* (Itaquasconinae) as an outgroup. ML bootstrap support values are presented below tree branches.

Shortened description of *Ramazzottius* cf. *conifer*: The original description of *H. conifer* appears brief and outdated when compared with the standards of modern tardigrade taxonomy. Thus, a redescription based on specimens from *terra typica* in Slovenia is desirable. However, with the lack of such material, we provide basic morphometric data for two individuals and three eggs from Scotland that we identified as *R.* cf. *conifer*. We designated the Scottish specimens as an uncertain identification since the redescription of *R. conifer* is lacking and because we were unable to observe the caudal cuticular papillae described by Mihelčič (1938). Thus, it is possible that the Scottish specimens do not represent *R. conifer* but a related species (although it also possible that the reported papillae are a sex-specific trait or simply a preparation artefact). Nevertheless, even if the Scottish specimens indeed represent a related species, rather than *H. conifer s.s.*, the analysis allows us to amend the generic classification of *H. conifer* as two closely related species that exhibit very similar morphology must belong to the same genus (in this case—the genus *Ramazzottius*). Until a proper redescription of *R. conifer* is available, the following data should be used with a certain dose of caution.

Animals: Body small, elongated (244–257 µm long, covered with smooth cuticle (Fig. 32). Conical pairs of papillae: one in the most caudal part of the body, and proximal and distal papillae on the fourth pair of limbs, absent or not visible. Buccal tube 22.7–25.5 µm long, very narrow (1.6–1.7 µm, *6.3–7.5%*). Stylet supports inserted at 13.7–15.1 µm (*59.2–60.4%*) of the buccal tube length. Small granular macroplacoids (Fig. 28): the first 3.0–3.2 µm long (*11.8–14.1%*), the second 2.3–2.4 µm long (*9.4–10.1%*). Macroplacoid row length 5.9–6.4 µm (*25.1–26.0%*). External and posterior claw lengths (Figs 33–34): bases 3.2–3.8 µm (*12.9–16.3%*), primary branches 8.4–12.2 µm (*37.0–53.7%*), and secondary branches 4.8–5.7 µm (*20.4–25.1%*).

Eggs: Slightly oval (smaller bare diameter 51.1–56.3 µm × larger bare diameter 54.7–61.8 µm), with regular rows of conical processes (10–15 processes per row, each 5.8–7.8 µm long), often underdeveloped (Fig. 35). Inter-

process distance 2.0–2.5 μm long, although process bases are sometimes connected to form a single row of processes (Fig. 36).



FIGURES 32–36. *Ramazzottius* cf. *conifer* **comb. nov.** (Mihelčič, 1938) from Scotland, seen in PCM: **32**—habitus, ventral view; **33**—claws I; **34**—claws IV; **35**—egg, note underdeveloped processes in the upper right part of the egg; **36**—the other side of the same egg, note rows of connected processes characteristic for the species. All scale bars in μm.

Discussion

Comparison with earlier descriptions of *H. dujardini.* The original description by Doyère (1840) of *Macrobiotus dujardini* was very limited compared to modern standards in tardigrade taxonomy (Michalczyk & Kaczmarek

2013). This was one of the first publications addressing tardigrade biology, and the two other formally described tardigrade species classified within *Macrobiotus* at the time were *M. ursellus* and *M. hufelandi*. Thus, Doyère (1840) only compared *M. dujardini* with these two species (N.B. *M. ursellus* is now invalid and *M. hufelandi* is classified in a different eutardigrade superfamily). Many of the traits that Doyère (1840) used for the differential diagnosis, such as the number of body cavity cells or the deposition of smooth eggs in exuviae, are currently considered either taxonomically irrelevant or relevant at higher taxonomic levels.

After the original description of *H. dujardini* several researchers published modernised descriptions of the species (Cuénot 1932, Marcus 1936, Bertolani 1982, Ramazzotti & Maucci 1983) or provided morphometric measurements to differentiate their new species from *H. dujardini* (Miller *et al.* 2005, Pilato *et al.* 2006, 2011, 2012). Importantly, however, none of these descriptions or measurements constituted a formal redescription based on material from the *locus typicus*. With the exception of Cuénot (1932), these researches based their descriptions or comparative morphometric measurements on specimens collected from a variety of localities far from the *locus typicus*; for example, Italy (Bertolani 1982), numerous sites throughout the globe (Marcus 1936, Ramazzotti & Maucci 1983), or undefined sites (Miller *et al.* 2005, Pilato *et al.* 2006, 2011, 2012).

Nearly a century after the original description of *dujardini*, Cuénot (1932) gave a more detailed description of *H. dujardini*, based on material from several localities in France. Although Fontainebleau was among the reported sites, Cuénot (1932) based his observations on several populations collected throughout France, thus it is not certain whether he based his description on a single or multiple species within the *dujardini* complex, since DNA sequencing that would allow an independent verification of the identifications was then not yet available. He noted well-marked granular eyes, elongated claws with short, narrow basal portions and eminent accessory points, two macroplacoids (the first with a slight constriction in the middle), and a tiny microplacoid ('comma'). He also stated that the species was aquatic and herbivorous. Marcus (1936) added a narrow bucco-pharyngeal tube (up to 2 μm) to the description and noted that the first macroplacoid is 1.5 times longer than the second. As a result, he synonymised numerous species described from around the world with *H. dujardini*, because their descriptions all matched the simplistic diagnostic criteria commonly adopted at the time. Bertolani (1982) stated explicitly that the species had a septulum not a microplacoid and he provided a detailed drawing of an Italian individual he classified as *H. dujardini* (figure 47 in Bertolani 1982). Ramazzotti & Maucci (1983) stressed putative problems with the distinction between *H. dujardini* and *H. convergens*. They pointed out the following differences between these two species: more slender and longer macroplacoids in *H. dujardini vs* more granular macroplacoids in *H. convergens* (compare Figs 3 and 26) and better marked 'microplacoid' and longer claws in the *H. dujardini* (compare Figs 5–6 and 27–28). However, they also classified specimens without the septulum from Greenland as *H. dujardini*, which today's modern taxonomic standards would most likely identify as a new species. Ramazzotti & Maucci (1983) defined the species as not strictly aquatic, but related to hydrophilic substrates.

Miller *et al.* (2005) and Pilato *et al.* (2006a, 2011, 2012) used individuals collected from undefined localities as a comparative material aiding descriptions respectively of *H. heardensis*, *H. seychellensis*, *H. pallidoides*, and *H. valentinae*. Miller *et al.* (2005) did not provide detailed morphometrics of the specimen they classified as *H. dujardini*, but the body length of 500 μm seems quite large and may indicate a new species (max 339 μm in the neotype series). Measurements of the individual that Pilato *et al.* (2006a and 2012) classified as *H. dujardini* (tables 2 in Pilato *et al.* 2006a, and 4 in Pilato *et al*. 2012; slide 4138 in the Binda and Pilato collection) differ substantially from the neotype series (Table 4). Pilato *et al.* (2006a and 2012) provided measurements of several traits for a single individual, but their specimen has larger placoids, septulum, and claws, both in absolute and relative (*pt*) terms (please compare respectively tables 2 and 4 in Pilato *et al.* 2006a and 2012 with Table 4 in the present study). These morphometric differences indicate a potential new species. Moreover, Pilato *et al.* (2006a and 2011) noted that the specimen they classified as *H. dujardini* had a peculiarly conical buccal tube, narrower towards the mouth opening (plate 1C in Pilato *et al*. 2006a, and figure 9 in Pilato *et al.* 2011; slide no. 2728 in the Binda and Pilato collection). However, in Pilato *et al.* (2012) a different specimen, also classified as *H. dujardini*, has a tube with an equal diameter throughout its length (figure 11D in Pilato *et al.* 2012; slide no. 4138 in the Binda and Pilato collection). We have never observed such anterior narrowing in any *H. dujardini* complex individuals, thus we hypothesise it may be a developmental aberration or a preparation artefact (see Morek *et al.* 2016b for effects of slide preparation on buccal tube diameter). However, if the narrowing is present consistently in a number of individuals, it may suggest a genuine qualitative trait and therefore a new species within the *H. dujardini* complex.

To conclude, it is important to stress that descriptions of *H. dujardini* by Cuénot (1932), Marcus (1936), Bertolani (1982) and Ramazzotti & Maucci (1983) cannot be considered reliable, as it is not possible to verify whether these authors based their observations on *H. dujardini* or on different species within the complex. Moreover, the individuals used by Pilato *et al.* (2006a, 2011, 2012) as a comparative material, supporting descriptions of *H. seychellensis*, *H. pallidoides*, and *H. valentinae*, differ morphometrically from the neotype *H. dujardini s.s.*, and most likely represent new species.

Geographic distribution of *H. dujardini*. Despite the lack of a detailed original description that would allow confident identification of *H. dujardini*, the species has been reported globally, with only the more recent reports acknowledging the species complex and using the uncertain *H.* cf. *dujardini* (e.g. McInnes 1994, Kaczmarek *et al.* 2014b, 2015, 2016, McInnes *et al*. 2017). However, some authors have noted that several earlier *H. dujardini* records from more remote localities do in fact represent new species within the *H. dujardini* complex (*e.g.* see Miller *et al.* 2005 for a discussion on Antarctic records of *H. dujardini*). Nevertheless, it is impossible to state which, if any, of the past records represent *H. dujardini s.s.* Our present study should, therefore, be considered as a reset point for the geographic distribution of the species. A similar approach was proposed for *Milnesium tardigradum* Doyère, 1840 redescribed by Michalczyk *et al*. (2012). The redescription aided the verification of some older records of *Milnesium tardigradum* that turned out to represent new species (*e.g.* Meyer *et al*. 2013, see also Morek *et al*. 2016a). Thus, we propose that, depending on the type of available data, the following identifications may be achieved:

H.* aff. *dujardini—when qualitative traits fit the redescription but there are no quantitative data, or the measurements diverge from the ranges described here (= an unidentified species of the *H. dujardini* complex).

H.* cf. *dujardini—when qualitative traits fit the redescription but incomplete quantitative data do not allow full verification of the identification against the neotype series (= a probable but uncertain record of *H. dujardini*).

H. dujardini—when qualitative and quantitative traits fall within the ranges described in this study and/or DNA sequences show immediate relatedness to the sequences provided here (= a certain record of *H. dujardini*).

Importantly, however, the striking phenotypic similarity of *H. dujardini* and *H. exemplaris* **sp. nov.** paralleled with considerable p-distances in all four analysed DNA markers suggest that species of the *H. dujardini* complex may be characterised by morphological stasis. In fact, the apparent differences between *H. dujardini* and *H. exemplaris* **sp. nov**. are limited to a different shape of the basal claw, cuticular bar shape and the *pt* of the SSIP, thus the two species could be easily mistaken by untrained researchers. In other words, the two species could be classified as pseudocryptic taxa. This implies that there could be species that are more closely related to *H. dujardini* and with no morphological or morphometric differentiating traits, *i.e.* true cryptic species. Therefore, we strongly suggest corroborating future *H. dujardini* records with molecular markers, even if the specimens fit the redescription perfectly. This will eventually lead to establishing the extent of intraspecific phenotypic and genetic variation and, as a consequence, verify the authentic geographic range of the species. Such data are still scarce for tardigrades, but the few available studies (Jørgensen *et al.* 2007, Cesari *et al.* 2016, Gąsiorek *et al.* 2016) suggest that *H. dujardini* may also have a limited geographic distribution. If this is so, then records outside the Holarctic or even Palaearctic will most likely represent new species within the *H. dujardini* complex. Currently, the only confident statement on the distribution of *H. dujardini* is that it was described from western Palaearctic. There is the potential that some of the *H. dujardini* synonyms, especially those globally distant from the *locus typicus*, may in fact be valid species; though insufficiently described and requiring thorough revision (*e.g. Macrobiotus murrayi* Richters, 1907, *Macrobiotus samoanus* Richters, 1908, *Macrobiotus breckneri* Richters, 1910).

Polyphyly of *Hypsibius*. *Hypsibius*, the fourth established tardigrade genus, initially comprised numerous hypsibioid and non-hypsibioid phyletic lineages that shared one common characteristic, *i.e.* a superficial resemblance of claw morphology. The claws were clearly different from those of macrobiotids and apochelans, thus the variability in claw morphology within the original genus was neglected. Nevertheless, in subsequent years, new genera abundant in species were erected, such as *Diphascon* Plate, 1888 (comprising taxa with the pharyngeal tube), *Isohypsibius* Thulin, 1928 (distinguished on the basis of different claw anatomy), or *Ramazzottius* Binda & Pilato, 1986 (characterised by elongated primary claw branches and cephalic elliptical organs). Consequently, though initially one of the largest eutardigrade genera, the group shrank gradually to the current 42 species and it is expected that several more genera will be isolated from the *Hypsibius* genus.

The scarcity of suitable molecular data for the majority of species comprising *Hypsibius* hinders the resolution of phyletic affinities within the genus. However, the 18S rRNA hypsibiid and the COI hypsibiin phylogenetic trees clearly indicate *Hypsibius* is polyphyletic, which is in agreement with the results obtained by Bertolani *et al*. (2014). For example, *H. scabropygus* (which probably represents a species complex; Zawierucha *et al.* 2014), appeared as the sister taxon to other Hypsibiinae in the 18S rRNA analysis, and shares many important taxonomic traits with the genus *Ramazzottius*, *i.e.* has two granular macroplacoids, elongated primary branches of posterior claws, and sculptured dorso-caudal cuticle. Furthermore, *H. klebelsbergi*, inferred as the sister group to *Borealibius* + *Hypsibius s.s*. clade in COI analysis, has pigmented body and strongly reduced, robust claws of a modified *Hypsibius* type (Dastych *et al*. 2003). Therefore, taking into consideration the clear morphological autapomorphies and evident genetic distinctiveness of *H. scabropygus* and *H. klebelsbergi*, we foresee further research raising two new genera within the current *Hypsibius*. Taking this onto consideration, it would not be surprising if future research reduces the genus *Hypsibius* to just the *H. dujardini* and *H. convergens* groups.

Taxonomic key to the *dujardini* group species

Definition: *Hypsibius* spp. with smooth cuticle, and two macroplacoids and a clear septulum in the pharynx.

Generally, structure ranges given by previous authors refer to adult individuals (second instar onwards, *ca.* >200 μm in body length). As absolute values can be significantly different for juveniles, we recommend that only adults are identified. Juvenile identification to species level in the *dujardini* group is currently impossible, as juvenile morphometric data are only available for a few of the described species.

1.	Cuticular bars on legs I–III present	2
-	Cuticular bars on legs I–III absent	4
2(1).	Septulum longer than 1.5 μm	3
-	Septulum no longer than 1.0 μm	*H. heardensis* Miller *et al.*, 2005
3(2).	The *pt* of SSIP higher than *64.0%*	*H. septulatus* Pilato *et al*., 2004
-	The *pt* of SSIP lower than *62.5%*	*H. conwentzii* Kaczmarek *et al*., 2018
4(1).	The *pt* of SSIP higher than *65.5%*	***H. exemplaris* sp. nov.**
-	The *pt* of SSIP lower than *64.5%*	5
5(4).	The *pt* of SSIP higher than *57%*	6
-	The *pt* of SSIP lower than *56%*	*H. pallidoides* Pilato *et al*., 2011
6(5).	External and posterior primary claw branches longer than 14 μm	*H. valentinae* Pilato *et al*., 2012
-	External and posterior primary claw branches shorter or equal to 14 μm	7
7(6).	The *pt* of the external buccal tube width higher than *6.5%*, *pt* of the septulum length below *7%*	***H. dujardini* s.s. (Doyère, 1840)**
-	The *pt* of the external buccal tube width lower than *6.5%*, *pt* of the septulum length above *7%*	*H. seychellensis* Pilato *et al*., 2006

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